# MOLECULAR ASPECTS OF B LYMPHOCYTE DIFFERENTIATION

*Organizers: Randolph Wall and Paul Kincade*

February 1 - 8, 1993; Taos, New Mexico

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# Molecular Aspects of B Lymphocyte Differentiation

*Keynote Address (Joint)*

**FZ 001** GENETIC ANALYSIS OF B CELL DEVELOPMENT, Klaus Rajewsky, Institute for Genetics, University of Cologne, FRG.

B cell development is governed by cellular selection through surface immunoglobulin. This principle operates at various stages of development. When B cells develop from stem cells in the bone marrow, cells failing to assemble their immunoglobulin V region genes properly are rigorously eliminated. Gene targeting experiments have been instrumental in advancing our knowledge of the sophisticated mechanisms which control this process and allow the production of a cell population expressing a selected antibody repertoire which is further shaped by interactions of the cells with their environment. Similarly, when memory B cells are generated in T cell dependent responses in germinal centers through somatic hypermutation of their V region genes, only cells expressing Ig receptors of suitable antigen binding specificity are permitted to survive. This process of positive and, perhaps, negative selection has striking analogies to the maturation of T cells in the thymus. It can now be approached through the isolation of single B cells from various, histologically defined positions in the microenvironment of the germinal center and their subsequent molecular analysis.

*Stem Cells and Early B Cell Progenitors (Joint)*

**FZ 002** STROMAL CELL CONTROL OF B CELL DEVELOPMENT. Shin-Ichi Nishikawa[1], Takumi Era[1], Minetaro Ogawa[1], Shin-Ichi Hayashi[1], Takahiro Kunisda[1], Satomi Nishikawa[1], Richard. R. Hardy[2], Yuji Yamanashi[3], Tadashi Yamamoto[3], Werner Müller, Klaus Rajewsky[3], [1]Institute of Molecular Embryology and Genetics, Kumamto University School of Medicine, Kumamoto, Japan, [2]Fox Chase Cancer Research Institute, Philadelphia, USA, [3]Institute of Medical Science, Tokyo University, Tokyo, Japan, [4]Institute for Genetics, Cologne University, Cologne, FRG

Intramarrow B cell-genesis is regulated by fibroblastoid stromal cell component in mouse bone marrow. The fact that a stromal cell clone, ST2 is capable of supporting the entire process from single FACS-sorted multipotent stem cells into sIgM$^+$ B cells clearly indicates that all molecules required for this process is expressed by ST2. We have been investigated the molecular basis for stromal cell-dependent B cell genesis. Among a number of cytokines which are constitutively or inducibly expressed by ST2, IL-7 and the ligand for c-kit (Steel Factor, SLF) are indispensable for B cell-genesis. However, while no active B cell genesis occurs in the IL-7(-) microenvironment, a considerable B cell-genesis is still observed in the SLF(-) microenvironment. Thus, it is likely that some other stromal cell molecule(s) may compensate for SLF, although SLF is essential for actual intramarrow B cell-genesis. When both SLF and IL-7 are included in the culture of the earliest B progenitor cells, proliferation and maturation of B lineage cells are supported. However, the extent of B lineage cell proliferation in such a culture is far less than that in the culture with stromal cell layers. This suggests that the actual B cell-genesis is regulated by multiple cytokines, among which some, inclucing IL-7 and SLF, support basal cell proliferation, while others may modulate this basal level. Nonetheless, using recombinant IL-7 , SLF and IL-7(-) stromal cell clone, PA6, the process of early B cell-genesis is divided into three stages with different growth signal requirements. Differentiation stages of B cell development has been defined also either by the organization and expression of immunoglobulin genes or by the expression of a number of surface molecules. We next attempted to correlate the differentiation stages in terms of growth signal requiremnts with the differentiation stages defined by other criteria. While some loose correlation was present between the growth signal requiremnts and the surface differentiation markers, it could hardly be a strict correlation. Thus, the signals regulating the growth signal receptors, surface differentiation markers, and immunoglobulin gene rearrangement might be different, though overlaps to a considerable extent. To gain an insight into the signalling pathways regulating the B lineage cell-differentiation, we have isolated $\mu^-$ B cell clones which apoptose by the LPS-stimulation. The similar apoptosis is inducible by introducing activated form of *lyn* into the same cells. Using these cell lines, the molecules which are able to rescue this cell death is currently screened. Finally, the studies of ours and other groups indicate that the expression of SLF is the minimum requirements for the hematopoietic as well as B-lymphopoietic microenvirioments. To understand the molecular basis for tissue-specific and stage-specific expression of murine SLF gene, we cloned the genomic SLF gene and determine a region which renders it expressed in fibroblastoid lines but does not so in hematopoietic cell lines. The same region was ligated to bacterial β-gal gene and introduced to fertilized eggs, which eventually gave rise to a number of transgenic mouse strains. Since these mice express β-gal gene in a tissue specific manner, they will provide a powerful tool to investigate the necessary conditions which modulate the stem cell vs stromal cell-interaction in hematopoietic tissues as well as other c-kit-dependent stem cell systems.

**FZ 003** GROWTH FACTOR REGULATION OF LYMPHOHEMOPOIETIC PROGENITORS, Makio Ogawa, Ralph H. Johnson Department of Veterans Affairs and Department of Medicine, Medical University of South Carolina, Charleston, South Carolina.

In the steady state marrow, the majority of hemopoietic stem cells are dormant in the cell cycle and only small numbers of stem cell clones provide all of the blood cells. During regeneration from marrow aplasia these cells are induced to proliferate actively by a group of early-acting cytokines. By serial observations of blast cell colony development, we have demonstrated that combinations consisting of either IL-3 or steel factor (SF) and one of IL-6, G-CSF, IL-11 or IL-12 (natural killer cell stimulating factor, cytotoxic lymphocyte maturation factor) effectively support proliferation of the primitive progenitors. In this communication, we present that similar cytokine combinations are also involved with the proliferation of lymphohemopoietic progenitors in culture

The existence of pluripotent lymphohemopoietic stem cells was postulated for many years through clinical observations such as identification of chromosomal markers in myeloid as well as lymphoid cells in the recipients of bone marrow transplantation and identification of the Philadelphia chromosome in myeloid and lymphoid cells in patients with chronic myelogenous leukemia. Recently, studies using retroviral labeling of individual hemopoietic progenitors confirmed the existence of the population of cells that are able to provide both myeloid and lymphoid cell lineages. Despite these *in vivo* evidence, it has not been possible to detect and quantitate the lymphohemopoietic progenitors until recently because of the absence of appropriate cell culture techniques. Now investigators in 2 laboratories have described culture assays for murine and human fetal bone marrow lymphohemopoietic progenitors by co-culture with murine marrow stromal cells. In our laboratory, we described a two-step methylcellulose culture system in which we unequivocally demonstrated that individual primitive progenitors from 5-fluorouracil-treated mice have a capacity for differentiation in both myeloid and B-cell lineages. By using micromanipulation techniques we plated highly enriched primitive murine marrow cells individually in the presence of pokeweed mitogen spleen conditioned medium (PWM-SCM), SF, erythropoietin and IL-7. Resulting primary myeloid colonies were replated on day 11 of culture into secondary cultures containing SF and IL-7. Approximately 1/4 of the primary micromanipulated cells yielded pre-B cell colonies in secondary cultures. When pre-B cell colonies derived from a single lymphohemopoietic progenitor were pooled and individually injected into scid mice, donor-type IgM was detected in the serum of the mice and the spleens contained donor-type B-cells upon sacrifice on day 112. We then tested growth factor combinations that might replace PWM-SCM in the primary culture. Combinations of two factors that included SF together with IL-6, IL-11, G-CSF or IL-12 effectively supported the B-lymphoid potential of the primary myeloid colonies. Colonies supported by combinations of IL-3 plus one of the synergistic factors failed to maintain the lymphoid potential. Our observations demonstrated that many cell-cycle dormant progenitors from 5-fluorouracil-treated mice which were previously believed to be myeloid-committed, also possess B-lymphoid potential. Studies of the mechanisms regulating early-stages of lymphohemopoiesis should be facilitated by this culture system.

*B Cell Adhesion and Accessory Molecules (Joint)*

FZ 004 B CELL TRIGGERING VIA CD38, Maureen Howard, Leopoldo Santos-Argumedo, Christopher Grimaldi, Fernando Bazan, Nobuyuki Harada and Michael Parkhouse†. DNAX Research Institute, Palo Alto, CA 94304 and †Institute for Animal Health, Pirbright Laboratory, Surrey GU24 ONF, England

We have recently isolated a cDNA encoding a murine B cell activation antigen initially identified by a growth-inducing monoclonal antibody. Sequence analysis of this cDNA revealed it was a novel murine protein exhibiting 70% homology to human CD38. Surprisingly, this putative murine CD38 exhibits a cellular distribution that is markedly disparate from that of human CD38. While that latter is expressed on human T cells, immature B cells, germinal center cells and plasma cells, the putative murine CD38 is expressed almost exclusively on all unprimed mature murine B cells. Triggering murine B cells with an agonistic anti-CD38 antibody in the presence of IL-4 induces $Ca^{++}$ mobilization and proliferation. This proliferation is augmented by LPS or anti-CD40 antibodies, but not by anti-IgM antibodies. Recent structural analyses have suggested CD38 is related to an enzyme involved in a recently discovered pathway of $Ca^{++}$ mobilization that is distinct from the inositol 1,4,5 triphosphate pathway of $Ca^{++}$ mobilization.

FZ 005 STRUCTURE AND FUNCTION OF LYB-2/CD72, Jane R. Parnes, William H. Robinson, Han Ying, Ju-Fay Chang and Henry N. de Vegvar, Stanford University Medical Center, Stanford.

Lyb-2/CD72 is a 45-kDa cell surface glycoprotein expressed on early B lineage cells through mature B cells, but not on terminally differentiated plasma cells. Early functional studies had demonstrated that monoclonal antibodies specific for mouse Lyb-2/CD72 could block differentiation into plasma cells in response to T cell-dependent antigens and could induce B cell proliferation. We have isolated and sequenced cDNA clones encoding Lyb-2/CD72 in both mouse and man, and these studies have led to the conclusion that Lyb-2/CD72 is a type II membrane protein homologous to CD23 and asialoglycoprotein receptors, all of which belong to the C-type lectin superfamily. We have sequenced cDNA clones encoding three serologically defined alleles of mouse Lyb-2/CD72. These studies have demonstrated that the alleles are highly conserved in their cytoplasmic and transmembrane domains but exhibit a high degree of polymorphism in their extracellular domains. This polymorphism in the extracellular region involves amino acid substitutions at a minimum of 20 residues and is concentrated primarily in the membrane distal region, the region that is also least conserved between mouse and human Lyb-2/CD72. We have demonstrated that mouse and human Lyb-2/CD72 each are ligands/receptors for both mouse and human CD5, a cell surface glycoprotein expressed on all T lymphocytes and a subset of B lymphocytes. We are currently examining how this binding affects B cell/T cell interactions. To further understand the role of Lyb-2/CD72 in vivo we have generated transgenic mice in which the Lyb-$2^a$ allele is expressed in an Lyb-$2^b$ mouse under the control of the immunoglobulin kappa light chain promoter and enhancers. This should lead to expression of both the Lyb-2/CD72 transgene and the endogenous gene on mature B cells, but while expression of the endogenous gene is turned off upon differentiation into plasma cells, expression of the transgene should increase. We have demonstrated transgene expression is indeed restricted to $B220^+$ cells in peripheral blood, making this vector potentially useful for the study of other transgenes in B lineage cells. Further analysis of the phenotype of these transgenics will be presented.

FZ 006 THE ROLE OF CD22 IN LYMPHOCYTE ADHESION, Ivan Stamenkovic[1], Dennis Sgroi[1], Sten Braesch-Andersen[1], Ajit Varki[2] and Alejandro Aruffo[3], [1]Department of Pathology, Massachusetts General Hospital, and Harvard Medical School, Boston, [2]Department of Molecular Medicine, University of San Diego, La Jolla and [3]Bristol Myers Squibb Pharmaceutical Institute, Seattle.

CD22 is a B cell-specific adhesion molecule expressed on mature B cells as two isoforms of 130 and 140 kD. Expression of CD22 occurs in the cytoplasm early in B cell ontogeny and on the surface at about the same time as surface IgD. Surface expression of CD22 coincides with acquisition of B cell responsiveness to antigen, suggesting that it may play a role in early B cell activation events which are T cell-dependent. cDNAs encoding two isoforms of CD22 have been isolated, and the predicted amino acid sequence shown to contain 5 and 7 Ig domains in the extracellular region of the smaller ($\alpha$) and larger ($\beta$) isoform respectively. Introduction of CD22$\alpha$ into COS cells promotes rosetting of erythrocytes and monocytes, whereas COS cells transfected with CD22$\beta$ bind T and B cells in addition to monocytes and erythrocytes. The first three domains of CD22$\beta$ contain the sequences necessary for recognition of ligands on adjacent cells. A soluble CD22-Ig fusion protein was used to identify ligands of CD22, and was found to immunoprecipitate multiple cell surface glycoproteins from both T and B cells, the major species being 115, 130 and 180-220 kD molecules. Immunoblotting experiments revealed that the high molecular weight bands correspond to different isoforms of CD45, the leukocyte common antigen. To determine whether interaction between CD22 and its T cell ligands might have an effect on T cell activation, T cells were stimulated by CD22Ig crosslinked with anti-CD3 antibody. A dramatic inhibition of intracellular calcium mobilization produced when T cells are stimulated with anti-CD3 alone was observed. Similarly, inhibition of PLC$\gamma$1 phosphorylation was also observed, closely reminiscent of the effect of coligating CD3 and CD45. CD22 interaction with CD45 and other cell surface ligands is dependent upon the presence of ligand-associated sialic acid in $\alpha$2,6 linkage. Transfection of COS cells with a cDNA clone encoding $\alpha$2,6 sialyltranferase alters the COS cell phenotype and promotes reactivity with CD22Ig. CD22Ig immunoprecipitates a 115 kD glycoprotein from $\alpha$2,6ST-transfected COS cells but not from mock-transfected counterparts. CD22 therefore behaves as a sialic acid binding lectin, and is the first Ig-like molecule to be observed to do so. It many play an important role in T cell-B cell interaction by helping regulate T cell responses.

**FZ 007** MULTIMERIC CD19-CELL SURFACE PROTEIN COMPLEXES REGULATE B CELL FUNCTION, Laura E. Bradbury[1], Liang-Ji Zhou[1], Shoshana Levy[2], Robert L. Evans[3], and Thomas F. Tedder[1], [1]Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, [2]Stanford University School of Medicine, Stanford, CA, [3]Roswell Park Memorial Institute, Buffalo, NY.

CD19 noncovalently associates with CD21 and a number of other surface proteins to form a multimolecular signal transducing complex. CD19 is a member of the Ig superfamily and CD21 is a member of the complement receptor family. Both are only expressed by B cells and follicular dendritic cells. CD19 is expressed from the early stages of Ig heavy chain rearrangement, and CD21 is first expressed at the time of IgD expression. Two additional members of the CD19 complex have been identified as TAPA-1 and Leu-13, which are physically associated in many cell lineages. TAPA-1 is expressed on most cell types while Leu-13 is expressed on subsets of lymphoid cells. Precipitation of CD19 specifically coprecipitates CD21, TAPA-1 and Leu-13 from B cell lines lysed in digitonin buffers. Western blot analysis with a TAPA-1 mAb verified the identity of TAPA-1 in CD19 and CD21 immunoprecipitated materials. In addition, when TAPA-1 or Leu-13 were crosslinked and patched on the cell surface, CD19 comigrated with them. Furthermore, mAb binding to CD19, CD21, TAPA-1 and Leu-13 on B cell lines induced similar biologic responses, including the induction of homotypic adhesion, inhibition of proliferation, and an augmentation of the increase in intracellular $Ca^{++}$ induced by suboptimal crosslinking of surface Ig on B cell lines. Together, these data suggest that TAPA-1 and Leu-13 are broadly expressed members of a signal transduction complex in which lineage-specific proteins, such as CD19 and CD21, provide cell specific functions.

CD19 has two extracellular Ig-like domains, a transmembrane domain, and a highly charged ~240 amino acid cytoplasmic tail. Significant evolutionary pressure for the conservation of amino acid sequences within the cytoplasmic tail suggests that it has a critical role in CD19 function. Therefore, the role of the cytoplasmic domain in signal transduction and the formation of intermolecular associations was examined using a series of cytoplasmic tail deletion mutants and a chimeric fusion protein containing only the extracellular portion of CD19. Deletion of all but the first 11 amino acids of the cytoplasmic tail had no affect on the ability of CD19 to associate with TAPA-1 and Leu-13, but the CD19 transmembrane domain was required. In addition, the induction of homotypic adhesion through CD19 or CD19 deletion mutants was equivalent, although homotypic adhesion was not induced through the CD19 chimeric molecule lacking the CD19 transmembrane and cytoplasmic domains. In contrast, growth arrest or $Ca^{++}$ flux induced through CD19 was decreased, but not ablated, by removal of the last 117 amino acids of the cytoplasmic tail. Thus, although all the CD19 deletion mutants retained the ability to associate with the other members of the CD19 complex on the cell surface, some, but not all, signaling events were impaired. Therefore, CD19 may regulate cell growth and $Ca^{++}$ flux via the cytoplasmic tail and other intracellular signals via its associated proteins.

To further examine the *in vivo* significance of CD19 expression, a series of eight transgenic mouse strains were produced that express differing levels of CD19. Ectopic expression of the human CD19 gene in transgenic mice was completely B lineage-restricted. As occurs in humans, CD19 was only expressed by B cells and was expressed by all mature B cells in blood and spleen. Each mouse line expressed varying amounts of human CD19, ranging from low to high levels of expression, depending on gene copy number. In general the phenotype of these mice was normal with one major exception. There was an inverse correlation between high expression of human CD19 and the number of circulating B cells in blood. This may correlate with increases in the number of B cells found in other tissues. It is possible that CD19 either directs the migration of B cells or that overexpression of CD19 makes B cells more susceptible to factors/stimuli that recruit them from the circulation. Further characterization of the unique features of these mice in conjunction with additional *in vitro* studies should provide considerable insight into the function of the CD19 signal transduction complex.

## *Developmental Microenvironments and Cell Lineages*

**FZ 008** MOLECULAR AND PATHOPHYSIOLOGIC CONTROL OF VH GENES IN MOUSE AND MAN, Lee A. Goodglick, Linda Berberian, Yadira Valles-Ayoub, and Jonathan Braun, School of Medicine, Jonsson Comprehensive Cancer Center, and Molecular Biology Institute, UCLA, Los Angeles, CA.

Only 10-20% of available germline V genes are commonly utilized in the human or murine antibody repertoire. Antigenic selection only partially accounts for this V gene pattern, and we have been interested to test whether cis-regulatory elements present in these V genes may contribute to their favored use. We selected the murine VH11 gene, which is exceptionally favored (>200-fold) in the B1 cell population. Through EMSA, footprinting, and competition studies, we were surprised to find that the likely site for this cis-regulatory element is in the leader exon encoded by a novel embedded octamer sequence. Functional studies with CAT constructs revealed that this leader segment indeed displayed enhancer activity, interestingly restricted to VH11+ cell lines. The presentation will describe the unusual features of the natural VH11 transcription unit, identification of the associated OCT isoforms, and efforts to functionally test their role in natural populations during VDJ rearrangement and Ig expression.

Human VH3 genes are notable for their striking restriction in abundance and anatomic distribution during ontogeny and development. One implication of this finding is that VH3 B cells may be sensitive clonal indicators of normal or pathologic immune processes. This idea has proven fruitful in studies of inflammatory bowel disease and acquired immunodeficiencies. In detailed studies of the 20p1 VH3 gene, we have determined that B cells expressing this gene are enriched in the colonic lamina propria, with a predominant antigenic specificity for an uncommon membrane glycoprotein species. We have found that 20p1 B cells are remarkably elevated (~20% total B cells) in inflamed mucosa of inflammatory bowel disease. This suggests that 20p1 antibodies may be a natural surveillance mechanism for colonic epithelial damage, and a marker or participant in autoimmune colonic disease. We have found the independent occurrence of clonal deletions of VH3 B cells in common variable immunodeficiency and HIV infection. In the context of AIDS, this finding is notable since AIDS is classically related to depletion of T cells bearing the HIV gp120 ligand, CD4. Analogously, we have recently found that ~10% of normal VH3 Ig is an avid ligand for HIV gp120. This suggests that certain VH3 Ig gene-products represent a second natural ligand for HIV, and we will present some of the implications with respect to HIV pathogenesis and vaccine design.

**FZ 009** IDENTIFICATION OF STROMAL CELL DERIVED SOLUBLE MEDIATORS THAT REGULATE PRIMARY B LYMPHOPOIESIS, Kenneth Dorshkind[1], Ramaswamy Narayanan[2], and Kenneth S. Landreth[3], [1]University of California, Riverside, CA 92521, [2]Hoffmann-La Roche Inc., Nutley, New Jersey 07110, [3]West Virginia University, Morgantown, West Virginia 26506.

During the initial stages of primary B lymphocyte development, pro-B cells develop into pre-B cells identified by their expression of the cell surface B220 molecule and cytoplasmic mμ (cμ) heavy chain protein. This process occurs in the bone marrow in association with a supporting network of stromal cells that regulate hemopoiesis. Our laboratories have characterized several stromal cell lines cloned from the adherent layer of long-term bone marrow cultures in order to further elucidate how the stroma regulates pre-B cell development, and these studies have revealed that soluble mediators play an important role. Studies, which have focused on one stromal cell line, S17, have revealed that conditioned medium from these cells can potentiate the formation of cμ expressing pre-B cells in which kappa light chain genes are rearranged but not transcribed. This S17 activity is distinct from Interleukin-7 (IL-7) and c-Kit Ligand (KL). Based on findings that recombinant Insulin-Like Growth Factor-I (IGF-I) can replace S17 conditioned medium in potentiating formation of pre-B cells and treatment of S17 stroma with an IGF-I anti-sense oligonucleotide results in marked inhibition of the pre-B cell activity, we have concluded that the factor responsible for these effects is IGF-I. This is consistent with previous observations indicating that the apparent MW of the activity is less than 10 kD. The data also indicate that while IGF-I alone does not have appreciable effects on proliferation of pre-B cells or their precursors, it acts to augment the growth effects of IL-7 once cells have differentiated to the B220+ stage of development. Taken together, these results have allowed formulation of a working model of primary B lymphopoiesis in which IGF-I potentiates formation of B220+ cells from B220− precursors. Whether this is due to direct stimulation of differentiation or is a survival effect which then allows cells to progress through a programmed pattern of development is not clear at this time. Subsequently, the B220+ progenitors/pre-B cells expand in response to IL-7, and this growth effect can be augmented by molecules such as KL and IGF-I, which alone do not stimulate cell growth.

FZ 010 B LYMPHOPOIESIS IN THE BONE MARROW: MICROENVIRONMENTAL ORGANISATION, REGULATION AND SELECTION, Dennis G. Osmond[1], Karen Jacobsen[1], Sergio Rico-Vargas[1], Lucy Fauteux[1], Homer Valenzona[1], Nancy Kim[1], Raffi Manoukian[1], Paul W. Kincade[2], Kensuke Miyake[2], Robert A. Phillips[3], Charles L. Sidman[4], and Shin-Ichi Nishikawa[5], [1]McGill University, Montreal, Canada, [2]Oklahoma Medical Research Foundation, Oklahoma City, [3]Hospital for Sick Children, Toronto, Canada, [4]University of Cincinnati, Cincinnati, [5]Kumamoto University, Kumamoto, Japan.

Stages in the differentiation of B lymphocyte precursors in mouse bone marrow have been defined by immunofluorescence labeling of terminal deoxynucleotidyl transferase (TdT), B220 glycoprotein and cytoplasmic or surface $\mu$ chains ($c\mu$, $s\mu$). A series of mitotic pro-B cells before the expression of $\mu$ chains ($TdT^+B220^-$, $TdT^+B220^+$, $TdT^-B220^+$) and of large pre-B cells ($c\mu^+s\mu^-$) give rise to non-dividing small pre-B cells and B lymphocytes ($s\mu^+$). In vivo studies of mitotic activity reveal a proliferative expansion of successive pro-B and pre-B cell populations, followed, however, by much (75%) cell loss. These processes have been visualized in situ by the binding of intravenously administered $^{125}$I-labeled monoclonal antibodies (mAb) revealed by electron microscope radioautography. $B220^+$ precursor B cells proliferate in association with stromal reticular cells. Molecules detected at this cellular interface in vivo by the binding of radiolabeled mAbs include VLA-4 and its stromal cell ligand VCAM-1. These molecules also characterize the stromal cell interface with non-lymphoid lineages. Some $TdT^+$ cells and $B220^+$ cells express the c-kit receptor which interacts with a stromal cell ligand. Whereas anti-c-kit antibody administration depletes erythroid and granulocytic cells in the bone marrow, however, the proliferative activity of pro-B and pre-B cells is increased. Thus, c-kit interactions appear not to be obligatory for precursor B cell development; blocking c-kit-dependent hemopoiesis may enhance B cell genesis by increasing the access of precursor B cells to stromal cells. A newly recognized molecule expressed on certain stromal cells is restricted to areas of the membrane which interact with lymphoid precursors. This suggests that lineage-specific microenvironmental niches may be created on the surface of individual stromal cells. In vivo proliferation of pro-B and pre-B cells is stimulated by systemic administration of the stromal cell product, interleukin 7, and by conditions of intense macrophage activation (sheep red blood cell injection, malaria infection, peritoneal oil-induced granuloma). The latter conditions also enhance cell loss. The macrophage product, interleukin 1, has a dual effect on precursor B cell proliferation; stimulatory at low dose levels but suppressive at high doses. The cell loss of B lineage cells in vivo is associated with apoptosis and a rapid uptake by resident macrophages. The appearance of apoptotic $B220^+$ cells and macrophage deletion are greatly enhanced in anti-IgM treated mice in which all newly formed cells are deleted as they are about to express $s\mu$, mutant mice with severe combined immunodeficiency (scid) unable successfully to rearrange $V_H$ genes in which all late pro-B cells abort, and $E\mu$-myc transgenic mice in which the constitutive expression of myc in the B lineage causes both an increased proliferation of precursor cells and an increased degree of apoptosis before the development of a malignant clone. Thus, the number and size of B cell clones normally produced to maintain primary humoral immunity are influenced by a variety of microenvironmental and systemic factors and represent a delicate balance between the processes of proliferation and negative selection.

*Surrogate Light Chains and Immunoglobulin Associated Molecules*

FZ 011 STRUCTURE OF THREE IG-LIKE COMPLEXES WITH $V_{PREB}/\lambda_5$ SURROGATE LIGHT CHAIN AND THEIR POSSIBLE FUNCTIONS IN B CELL DEVELOPMENT, Fritz Melchers, Hajime Karasuyama, Dirk Haasner, and Antonius Rolink, Basel Institute for Immunology, Basel, Switzerland.

Two genes, named $V_{preB}$ and $\lambda5$, were found to be selectively expressed in precursor (pre) B lymphocytes of mouse and man. The two proteins encoded by the $V_{preB}$ and $\lambda_5$ genes associate with each other to form a light (L) chain-like structure, called the surrogate L chain. Surrogate L chains can form Ig-like complexes with three partners - heavy (H), or heavy-like chains - which are expressed on the surface of pre B cells at different stages of development from a committed progenitor to an immature $sIg^+$ B cell. We will review what is known of the structures of the $V_{preB}$ and $\lambda_5$ genes in mouse, and their relatives in humans, and describe their pattern of expression as RNA transcripts and as proteins, and their possible evolution. We will speculate on the possible structure of the surrogate L chain, and on the possible functions along the pathway of development of cells of the B lymphocyte lineage.

FZ 012 ASSEMBLY AND ORGANIZATION OF PRE-B AND B CELL RECEPTORS - Shiv Pillai[1], Bobby Cherayil[1], Caterina Chiodino[1], Kenneth MacDonald[1], Anand Bachhawat[1], Stephen Desiderio[2] and Craig Smucker[2]. [1] Massachusetts General Hospital, Harvard Medical School, Boston, MA 02129, [2]Johns Hopkins University School of Medicine, Baltimore.

Membrane immunoglobulin heavy chains ($\mu$m) associate in pre-B lymphocytes with two pre-B specific surrogate light chains, the $\omega/\lambda5$ and the $\upsilon$/vpreB proteins. The $\mu$m-surrogate light chain complex is, like the antigen receptor in B cells, non-covalently associated with the mb-1 and B29 proteins and with a src-family protein tyrosine kinase. This complex of at least 6 proteins constitutes the pre-B receptor. The pre-B receptor functions to deliver a survival signal to B lineage cells which have made in-frame rearrangements of their heavy chain genes. Cell survival is presumably sufficient to allow, by default, further differentiation of pre-B cells and allelic exclusion at the heavy chain locus.

Assembly of the pre-B and B cell receptor (both $\mu$ and $\gamma$ isotypes, which represent broadly the two structural categories of membrane Ig) have been examined in pre-B and B cell lines as well as by site directed mutagenesis and transfection into Cos cells and L cells. The role of two putative ER chaperones, p90 (a transmembrane phosphoprotein which is the product of the calnexin gene) and p82, in receptor assembly have been examined. Calnexin/p90 associates transiently with $\mu$m, and p82 associates with $\gamma$m, making contact with the transmembrane domains of the immunoglobulin heavy chains.

Although it has generally been assumed that mb-1 directly associates with $\mu$m, we provide evidence for the interaction of B29 with $\mu$ and $\gamma$. B29 in fact helps form a bridge between $\mu$m and $\gamma$m on the one hand and the src family B cell specific blk tyrosine kinase on the other. A major component of the interaction of B29 with $\mu$m and $\gamma$m is with the extracellular domains of these proteins. B29 does not associate with overexpressed EGF receptor or with c-abl but is capable of associating in the ER with the intracellular ltk kinase.

Although membrane immunoglobulins have long been considered to be "7S", it is clear that the actual receptors are much larger structures. Indeed in the case of membrane IgM it is unclear whether monomeric IgM receptors would be of sufficient affinity to actually respond to soluble antigens. In the case of the pre-B receptor an important issue (in the context of ligand independent signalling models) is whether the receptor is capable of constitutive aggregation and signalling. The higher order oligomeric structure of pre-B and B cell receptors will be discussed.

**FZ 013** FUNCTIONAL STUDIES OF THE B AND PRE-B CELL RECEPTORS. Kwang-Myong Kim, Gottfried Alber, Thomas Apel, Heinrich Flaswinkel, Peter Weiser and Michael Reth. Max-Planck-Institute für Immunologie, Freiburg, Germany

The B cell antigen receptor (BCR) is a complex between the membrane-bound Ig molecule and the transmembrane heterodimer Ig-α/Ig-β. We have studies the signaling function of BCR in different B cell lines. In particular we found that the BCR on the surface of the myeloma cell, *J558L still can* transduce signals. In these cells the tyrosine kinases lyn and PTK72 become activated after receptor cross-linking. The tyrosine kinase activation is also seen by the increased phosphorylation of several substrate proteins.

In comparison to the B lymphoma lines WEHI 231 and K46 the myeloma J558L has only a few dominant tyrosine kinase substrate proteins. This may facilitate the identification of these proteins. Some of the already known substrate proteins are the BCR subunits Ig-α and Ig-β which became strongly phosphorylated after BCR triggering in J558L cells. We also have found an interesting, signaling variant of J558L. In this variant Ig-α and Ig-β become rapidly phosphorylated after BCR activation while other J558L substrate proteins are not at all phoyphorylated or with a delay of 10 to 30 minutes. As in this variant the tyrosine kinases appear functionally normal the defect must lay in the failure of interaction between the kinase and its substrate proteins.

To test the signaling function of the BCR subunits we have expressed fusion protein of CD8 with the cytoplasmic tail of either Ig-α or Ig-β in the B lymphoma line K46. Cross-linking of these minimal receptor activates tyrosine phosphorylation and results in Ca mobilisation. Thus, the cytoplasmic sequences of the Ig-α and Ig-β protein have an important role in signal transduction from the activated BCR. We previously have identified a conserved sequence motif (YxxL motif) in the cytoplamsic tail of Ig-α and Ig-β which is also found in the signaling subunit of other receptors (TCR, FcR).
The YxxL motif was also found in the cytoplasmic sequence of two viral transmembrane proteins, namely gp30 of Bovine Leukemia Virus (BLV) and LMP2A of Epstein-Barr Virus (EBV). This may indicate that these viruses which both activate the cell growth of infected B lymphocyte can mimic BCR signaling function. Apart from its surrogate light chain consisting of λ5 and V pre-B the structure of the μm pre-B cell receptor is similiar to that of the BCR. We are currently analysing the signal transduction from the pre-B cell receptor. These signals are of interest because they control pre-B cell development as well as V gene rearrangements.

*Immunoglobulin Rearrangements (Joint) (Session Sponsored by Abbott Laboratories, Diagnostics Division)*

**FZ 014** HOW IS THE LYMPHOKINE-PRODUCING PHENOTYPE OF AN IMMUNE RESPONSE ESTABLISHED, William E. Paul, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892 USA

The protective value of immune responses is largely determined by the set of lymphokines produced by T cells in that response. CD4+ T cells can develop into cells that principally produce IL-2 and IFNγ or into cells that principally produce IL-4, IL-5, IL-6 and IL-10. In order to examine the factors that control which set of lymphokines will be produced, we carried out *in vitro* priming of CD4+ T cells from mice transgenic for genes for the α and β chains of the T cell receptor for peptide 88-104 of pigeon cytochrome C in association with the class II MHC molecule I-E$^k$. These cells produced little IL-4 or IFNγ upon initial *in vitro* challenge with 88-104 and appropriate antigen-presenting cells (APC) although they proliferated vigorously. Upon rechallenge 4 days later, they produced either IL-4 or IFNγ (and IL-2) depending upon the conditions during the priming culture. The key determinant of the lymphokine-producing phenotype was IL-4 itself. If IL-4 was present during the priming culture, the cells were excellent producers of Il-4 upon challenge but made little or no IFNγ or IL-2. By contrast, if IL-4 were omitted, the cells produced IFNγ and IL-2 but no IL-4. Varying the type of APC changed the magnitude of the stimulation but did not effect the pattern. The effect of IL-4 in determination of lymphokine-producing phenotype was verified by in vivo experiments in which mice were treated with anti-IL-4 at the time of priming. Such treatment diminished the production of IL-4 upon in vitro challenge with antigen and APC for at least 75 days. By contrast, anti-IL-4 administered at the time of secondary challenge did not diminish IL-4 production in cells from mice that had been primed in the absence of anti-IL-4. The effect of IL-4 determining lymphokine-producing phenotype was correlated with a potent inhibitory action of IL-4 on IL-2 and IFNγ production in response to stimulation of naive T cells with soluble anti-CD3 and APC, concanavalin A and APC or antigen and APC. This inhibitory effect of IL-4 was not observed on long term T cell lines, on T cell hybridomas or on transgenic T cells that had been primed with antigen and were restimulated with antigen and APC in the presence of IL-4. Although IL-4 failed to inhibit IL-2 and IFNγ production by naive T cells in response to immobilized anti-CD3 or to calcium ionophore and phorbol ester, studies indicated that its effect was not at the level of blocking the CD28/B7 accessory signals delivered through the interaction of B7+ APC with CD28+ T cells. These results establish that IL-4 present at the time of priming is the principal factor that determines lymphokine-producing phenotype of CD4+ T cells and establish an experimental system in which the mechanism of IL-4 action can be studied.

**FZ 015** ANALYSIS OF IG GENE REARRANGEMENT IN A TEST SUBSTRATE IN TRANSGENIC MICE, Ursula Storb, Peter Engler, Emily Klotz and Andrew Weng, Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637.

We have produced a transgenic mouse line with seven head-to-tail copies of an artificial V-J recombination substrate, pHRD. This test gene contains the heavy chain enhancer and metallothionein promoter. The methylation of the transgene is under the control of a dominant strain-specific modifier gene, *Ssm-1* (1). When the transgene is methylated (in C57BL/6), no recombination can be detected, but when it is unmethylated (in DBA/2), rearrangement is easily seen in spleen, bone marrow, lymph nodes and Peyer's patches, but not in thymus or nonlymphoid tissues (2). In mice with a partial methylation phenotype, rearrangement is mainly seen in hypomethylated copies. Furthermore, only unmethylated pHRD transgenes are transcribed, and only in lymphoid organs. Thus, methylation interferes with both transcription and rearrangement, but undermethylation alone is not sufficient for expression. The brain may be an exception and is under further investigation.

The pHRD test gene does not encode a selectable Ig or TCR. Therefore, the V-J joints should persist as a pool of randomly created rearrangements. Over 70, mostly different, V-J joints were sequenced from fetal liver and adult lymphoid organs. In the fetus, no N regions were detected, whereas 74% of the joints in the adult contained N regions (3). Thus, the absence of N regions in endogenous fetal Ig genes is apparently due to a mechanistic difference between fetal liver and early pre B cells, presumably caused by differing levels of terminal transferase.

Since the pHRD test genes in this transgenic line are aligned as seven identical head-to-tail copies it was possible to determine whether the spacing between rearrangement signal sequences plays a role in rearrangement. It was found that recombination occurs randomly between any two recombination signal sequences within the transgene array (2). The significance of this finding with respect to the mechanism of V(D)J recombination will be discussed.

*Supported by NIH grants HD23089 and AI24780. P.E., E.K. and A.W. were supported by NIH training grants CA09594, GM07183 and GM07197 respectively.*

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*Signal Transduction via the Immunoglobulin Receptor Complex*

**FZ 016** THE B LYMPHOCYTE ANTIGEN RECEPTOR: SPECIFICITY AND PHOSPHORYLATION DEPENDENCE OF INTERACTIONS OF RECEPTOR SUBUNITS WITH CYTOPLASMIC ENZYMES INVOLVED IN SIGNAL PROPAGATION. John C. Cambier, Chris Pleiman, Joachim Friedrich, and Marcus Clark, Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado, 80206.

The B lymphocyte antigen receptor functions in transmembrane transduction of signals leading to cell activation, proliferation and differentiation. The B cell antigen receptor complex is a hetero-oligomeric structure composed of antigen binding, mIg, and transducer-transporter substructures. The transducer- transporter substructure is composed of disulfide-linked dimers of Ig-α and Ig-β/γ subunits that are products of the mb-1(α) and B29 (β/γ) genes. Ig-γ is a truncated form of the predicted full length B29 gene product (Ig-β) which lacks ~30 C-terminal amino acid residues. While Ig-β is expressed in resting B cells and B cell blasts, Ig-γ is only seen in B-cell blasts where it represents as much as 50% of the total B29 gene product expressed.

Previous studies conducted in a number of laboratories have demonstrated that the antigen receptor complex associates with src--family kinases that are activated following receptor ligation. However, the site of interaction of these and other cytoplasmic effector molecules with receptor subunits is unknown. We have approached this question using fusion proteins composed of glutathione S-transferase and cytoplasmic tails of Ig-α or Ig-β as ligands to isolate binding proteins from B cell lysates. The cytoplasmic tails of Ig-α and Ig-β chains were found to associate with distinct sets of effector molecules. The Ig-α chain cytoplasmic domain bound to the src--family kinases lyn and fyn, phosphatidylinositol-3 kinase (PI-3 kinase) and unidentified 52, 53 and 38 kD phosphoprotein the cytoplasmic tail of Ig-β bound PI-3 kinase and unidentified 40- and 42-kD phosphoproteins. These associations did not require tyrosine phosphorylation of α and β, indicating that they probably do not involve SH2- phosphotyrosine interactions. The binding activity of both chains was mapped to a 26 amino acid sequence motif (ARH1) found in multiple receptors including T and B cell receptors for antigen, and various Fc receptors. Finally, we foundthat phosphorylation of specific tyrosine residues within this motif alters it's binding activity, decreasing binding to the above molecules but increasing binding of an unidentified 50kDa species. These findings indicate that the subunits act independently to activate distinct second messenger pathways and that ARH1 motif interaction with cytoplasmic effectors is dynamically regulated by tyrosine phosphorylation. These findings will be discussed in the context of antigen receptor function in normal, anergic and desensitized B cells.

**FZ 017** B CELL ACTIVATION AND INACTIVATION: MODULATION BY T CELLS, CYTOKINES AND CD45, Gerry G.B. Klaus, Sarah Parry and Robert Brines, Laboratory of Cellular Immunology, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK

Surface IgM and IgD receptors on B cells can deliver either inactivating (tolerising) or activating signals depending on the state of differentiation of the cells. The biochemical basis of such differential signalling is not well-understood, but it is clear that both positive and negative signals can be modulated by T cell-derived influences and also by engaging other molecules on B cells, such as CD45. We have been studying these interactions in several model systems. We find that the activation of immature B cells from neonatal mice by LPS is powerfully inhibited by both anti-$\mu$ and (in the case of $sIgM^+sIgD^+$ B cells) by anti-$\delta$ antibodies. This inhibition is partially (in the case of anti-$\mu$), or completely (with anti-$\delta$) reversed by IL-4. We are therefore investigating whether other T cell-derived signals will synergise with IL-4 to reverse negative signals in this system. We find that fixed preactivated T cells activate immature B cells as effectively as mature cells. It is now believed that this phenomenon is largely due to the ligand for CD40, which is expressed on activated $CD4^+$ T cells, and experiments investigating the role of this molecule in modulating tolerance induction are underway.

Another model which has been extensively used for studying B cell tolerance is the series of phenotypically immature B cell lymphomas (including WEHI-231 and CH33), which stop growing and undergo apoptosis when cultured with anti-$\mu$ antibodies. It was believed that these inhibitory effects are only elicited following ligation of sIgM receptors. We find that these lymphomas express the RB isoform of CD45, and not CD45RA. Moreover, extensive crosslinking of CD45 with a series of RB-specific MAb induces growth arrest of these cell lines as effectively as anti-$\mu$. This effect is not induced by MAb to Class I or Class II, nor is it seen in mature B cell lymphomas, whose growth is not inhibited by anti-Ig. CD45 is known to be required for signalling via the sIg complex, and may well regulate the activity of src-related protein tyrosine kinase(s) associated with the complex. We are therefore currently investigating whether engaging CD45 stimulates or inhibits the protein tyrosine phosphatase activity of the molecule and also the relationship between the inhibitory signals generated via sIg versus CD45.

*Discussion: Transgenic Animals and Gene Knockouts*

**FZ 018** ES CELL DERIVED MODELS TO STUDY B CELL DIFFERENTIATION, Yoichi ShinKai, Jainzhu Chen, Barbara Malynn, Jocelyne Demongeot, Fay Young, and Frederick W. Alt. Howard Hughes Medical Institute, The Children's Hospital, Harvard Medical School, Boston, MA

Genes that encode Ig and T cell receptor variable regions are assembled by VDJ recombinase. Tissue specific components of VDJ recombinase are likely encoded by the RAG-1 and RAG-2 genes. We created RAG-2 deficient mice by gene targeted mutation. These mice are viable but have a severe combined immunodeficiency due to total inability to initiate VDJ recombination and thereby generate mature lymphocytes. The VDJ recombination defect in pre-lymphocyte cell lines from homozygous RAG-2 mutants can be corrected by introduction of RAG-2 expression constructs. RAG-2 deficient mice have no obvious defect in any tissue or lineage other than lymphocytes. We conclude that RAG-2 function and VDJ recombination are required only for lymphocyte development. We have also used gene targeted mutation to study other issues regarding B cell development. Transcriptional enhancers within the Ig loci may also operate as recombinational enhancers to target associated gene segments for VDJ recombination. To test this notion, we replaced the endogenous Ig heavy chain enhancer with an expressed $neo^r$ gene; the mutated locus lost ability to undergo VDJ recombination. The N-myc gene is expressed in many early lineages including precursor B lymphocytes where its expression is regulated by the pre-B growth factor, IL-7. We created mice that carry a germline mutation in the N-myc gene; the homozygous mutation is an embryonic lethal at approximately E10.5. Therefore, to analyze potential roles in lymphocytes, we reconstituted normal blastocysts with homozygous mutant ES cells. Homozygous mutant ES cells were able to generate precursor B cells and their phenotype is currently under analysis.

*Cytokine Interactions with B Cells (Joint)*

**FZ 019** CYTOKINE CONTROL OF HUMAN B LYMPHOCYTE DIFFERENTIATION, Jacques Banchereau, Dominique Blanchard, Francine Brière, Serge Lebecque and Françoise Rousset. Schering-Plough, Laboratory for Immunological Research, Dardilly, France.

INTRODUCTION : Antigens breaking the cutaneous or mucosal barrier are rapidly taken up by Dendritic/Langerhans cells which migrate to the draining lymph nodes where they initiate the development of T and B cell reactions. Antigen specific B cells proliferate and differentiate into plasma cells. Some B cells migrate within the follicles to generate germinal centers where they expand, undergo somatic mutations, isotype switching, antigen selection and differentiation into either memory cells or plasmablasts secreting antibodies of higher affinity and specificity. In order to understand the mechanisms controlling these steps, we have studied the role of cytokines in the growth and differentiation of B cells activated in two different systems : the CD40 system and the CD3 system.

THE CD40 SYSTEM: Resting B cells proliferate when cultured in the presence of anti-CD40 and a fibroblastic cell line (L cells) expressing an IgG Fc receptor ($Fc\gamma R_{II}$/CDw32). Additional triggering of the sIg results in T cell-independent differentiation of B cells. Naive $sIgD^+$ B cells produce mostly IgM and isotype committed $sIgD^-$ B cells produce mostly IgG and IgA. IL-4 strongly stimulates the proliferation of B cells cultured in the CD40 system and factor dependent B cell lines could be maintained for up to 10 weeks. Single B cells can give rise to colonies of 50-500 cells within two weeks. IL-10 enhances short term B cell proliferation and combinations of IL-4 and IL-10 result in strong multiplication of B cells. Naive B cells undergo isotype switching towards IgE in response to IL-4. This is demonstrated by using the clonal growth of G8 idiotype positive single B cells and by sequencing of the $VDJC_H$ products of single daughter B cells. B cells secrete large amounts of IgM, IgG and IgA in response to IL-10 following differentiation into plasma cells. Naive $sIgD^+$ $sIgM^+$ B cells, which produce essentially IgM in response to IL-10, are induced to secrete both $IgA_1$ and $IgA_2$ following addition of TGFß. In contrast, TGFß suppressed the IL-10 mediated IgG, IgM and IgA secretion by $sIgD^-$ B cells. The CD40 system displays many of the features of B-cell immunopoiesis, including intense proliferation, isotype switching and plasma cell differentiation, but does not to induce somatic mutations.

THE CD3 SYSTEM: Some $CD4^+$ clones, activated with immobilized anti-CD3, induce growth and differentiation of resting B cells. The proliferation is limited because cells readily differentiate into non proliferating plasma cells. These clones also induced isotype switching in naive B cells. Addition of IL-4 antagonists to cultures result in inhibition of IgE production while the secretion of other isotypes and the proliferation is poorly affected. Addition of IL-2 antagonists inhibits B cell proliferation and production of IgG, A, M without inhibiting that of IgE. T cell clones activated for 24-28 h and washed free of cytokines fail to induce B cell proliferation and differentiation. Addition of IL-2 (but neither of IL-4 nor of IL-10) to such cultures results in strong B cell growth and Ig secretion and IL-10 further enhances these effects.

PERSPECTIVES : Identification of culture conditions yielding somatic mutations will represent a major step towards the "*in vitro* immunization" of naive B cells. This would ultimately permit us to generate human monoclonal antibodies of desired specificity and unavailable in the human repertoire.

**FZ 020** THE TNF/NGF SUPERFAMILY OF RECEPTORS AND THEIR LIGANDS, Raymond G. Goodwin, Richard J. Armitage, Douglas E. Williams, Brian Gliniak, Kenneth Grabstein, William C. Fanslow, Terry Farrah, Ian B. McAlister, Ben Falk, Terri Davis, Wenie S. Din, and Craig A. Smith, Immunex Research and Development Corp., Seattle

A growing superfamily of receptor molecules has recently emerged which includes the two receptors for TNF, the low affinity receptor for NGF, the B cell antigen CD40, the lymphoid activation antigens CD27 and CD30, the rat T cell antigen OX40, the murine T cell antigen 4-1BB, the surface antigen Fas, as well as the predicted protein products of several viral open reading frames. Homology between the various family members is primarily confined to their cysteine-rich extracellular domains. Ligands for these receptor-like molecules have been identified for the TNF and NGF receptors, and recently for CD40. The CD40 ligand, like pro-TNFα, is a type II membrane protein with sequence similarity in its extracellular domain to TNFα and TNFβ. Using the extracellular domain of CD30 linked to the constant domain of human IgG1 as a probe, we have identified a ligand (CD30L) on the surface of an induced murine T cell clone. A cDNA encoding this protein was isolated by a direct expression method, the sequence of which revealed the ligand to be a 239 amino acid type II membrane protein with significant homology in its C-terminal, extracellular domain to TNFα, TNFβ, and the CD40 ligand. These results suggest the emergence of a ligand superfamily parallel to the receptor superfamily with which they interact. A human CD30L cDNA isolated from a peripheral blood T cell library by cross hybridization encodes a protein of 234 amino acids which is 72% identical to its murine homologue at the amino acid level. Investigations of the possible role of CD30, a surface marker on Reed-Sternberg cells, and CD30L in the pathogenesis of Hodgkin's Disease will be discussed.

*Activation and Regulation of B Cell Gene Transcription*

**FZ 021** REGULATION OF MHC CLASS II GENES: PROMOTER OCCUPANCY IN VIVO. Laurie H. Glimcher and Catherine J. Kara. Department of Cancer Biology, Harvard School of Public Health and Harvard Medical School, Boston, MA 02115.

The expression of the class II antigens of the major histocompatibility complex is tissue specific, developmentally regulated in cells of the B lineage, and cytokine inducible. DNA sequences termed X1, X2, and Y, that are critical for the transcription of the class II genes have been identified through transfection studies and transgenic analysis. Previously (Science 252:709, 1991), we have shown by in vivo footprinting that these elements were occupied only in cells that express class II genes, in spite of the presence of promoter binding proteins in other cells. Class II deficient combined immunodefiency is a hereditary disease resulting in abrogation of transcription of the class II genes due to a defect in the transacting regulatory factor. Cell lines from certain CID patients lack factor binding at multiple sites in class II promoters in vivo. A mutation in one of the promoter binding proteins could explain this "bare" phenotype only if these factors bind cooperatively or in a temporal hierarchy. Alternately, the mutation could affect the configuration of the promoter within the MHC locus. Here we provide evidence that the factor(s) defective in class II deficient CID controls the accessibility of class II promoters within the environment of the MHC. The in vivo occupancy of wild-type and mutated class II promoter constructs was examined in stable transfectants of normal and CID-derived cell lines. The CID promoter phenotype could not be reproduced in a normal cell line by eliminating binding at any one promoter element, suggesting these factors bind independently, both spatially and temporally. In contrast, promoter occupancy was partially restored in two CID lines at a randomly integrated wild-type promoter, implying that the promoter is inaccessible to factors in its native environment but accessible when moved to another location in the genome.

**FZ 022** REGULATION OF TdT EXPRESSION DURING LYMPHOPOIESIS, Patricia Ernst, Beatrice Zenzie-Gregory, Kiersten Lo, Kyungmin Hahm, and Stephen T. Smale, Howard Hughes Medical Institute and Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA.

Lymphocyte maturation depends on the activities of numerous proteins that are expressed with a wide range of developmental patterns. Terminal transferase (TdT) is one protein important for lymphopoiesis, whose expression is tightly restricted to both early B cells and early T cells. Thus, through the study of TdT gene expression, we expect to identify regulatory proteins that play a role in controlling the maturation of both types of lymphocytes. Early experiments have demonstrated that the TdT promoter appears sufficient for lymphocyte-specific expression and falls into a class of promoters that lack TATA boxes.

The lymphocyte specificity of the TdT promoter is dictated largely by a control element called TdT D', located between -50 and -80 relative to the transcription start site. This element is essential for promoter activity in lymphoid cell lines and is capable of interacting with multiple proteins. We purified a protein called LyF-1, which is enriched in B and T lymphocytes and interacts with two adjacent sites in the D' element. LyF-1 also binds to the promoters for several other lymphocyte-specific genes, suggesting that it may be a commonly used transcriptional activator. In addition to LyF-1, members of the ets family of nuclear oncoproteins bind to an element in the D' region, directly overlapping the proximal LyF-1 binding site. Some of the ets members that bind to D', including ets-1 and fli-1, are expressed predominantly in B and T cells. Currently, our analysis suggests that both LyF-1 and an ets protein may be essential for TdT transcriptional activation.

We also are interested in further understanding the mechanisms of transcription initiation from promoters that lack TATA boxes. This goal is important for understanding gene regulation in lymphocytes because TATA boxes are absent in many or most lymphocyte-specific genes, such as the λ5, VpreB, mb1, and B29 genes. To this end, we are analyzing a core element in the TdT promoter, called an initiator (Inr), that appears to carry out the same functions as TATA. The Inr directly overlaps the transcription start site and acts in concert with upstream activators to direct high levels of accurate transcription. We and others found that the TATA-binding protein (TBP) is essential for transcription from promoters lacking a TATA box and containing an Inr. To address the role of TBP during Inr-mediated transcription, the nucleotide sequence 30 bp upstream from the TdT Inr was varied extensively and each resulting promoter was tested for transcriptional activity and for TBP binding. Efficient Inr-mediated transcription was found with most promoters, but promoter strength was strongly reduced by highly GC-rich sequences that possess the lowest affinity for TBP. This suggests that TBP must interact with the -30 region during transcription initiation mediated by an Inr and that most, but not all, sequences interact with an affinity that is sufficient for high levels of transcription. Interestingly, the authentic -30 region of the TdT promoter contains a sequence that is highly GC-rich and that promotes Inr-mediated transcription relatively poorly. This characteristic of the TdT promoter might be an important feature that contributes to appropriate transcriptional regulation in developing lymphocytes.

*Tolerance and Autoimmunity*

**FZ 023** TOLERANCE AND THE MEMORY CELL LINEAGE, Norman R. Klinman, Phyllis-Jean Linton, and Debra J. Decker, The Scripps Research Institute, La Jolla.

Common to all paradigms of B cell inactivation is the ability of concomitant T cell help to circumvent tolerance and lead instead to activation. This $T_H$ dependent bypass of B cell tolerance should pose few problems with respect to self-non-self discrimination among neonatal or adult primary B cells since, during the tolerance susceptible phase of their development, antigen primed T cells would rarely be present. However, the generation of memory B cells requires $T_H$ dependent stimulation of their progenitors. Therefore, both antigen and $T_H$ specific for that antigen should be present during the "second window" of tolerance susceptibility that accompanies memory B cell generation. Because of this, the activation vs inactivation of those newly generated memory B cells that have acquired anti- self reactivity as a by-product of somatic hypermutation can be viewed as a competition between the stimulating antigen, for which T cell help is available, and tolerogen (self), for which T cell help is not available.

The results obtained from fragment culture analyses of cell populations enriched for progenitors of memory B cells (depleted of AFC precursors), indicate that newly generating memory B cells are highly susceptible to antigen mediated inactivation. As with tolerance induction of immature primary B cells, the inactivation of memory B cells requires multivalent antigen (receptor interlinkage) and is hapten inhibitable. The inactivation of secondary B cells is less specific than that of primary B cells in that it can be accomplished by cross-reactive determinants. Preliminary assessments of the aforementioned competition between stimulation and inactivation of memory B cells indicate that stimulation may be dominant. Thus, when an immunogen ($T_H$ available) and a tolerogen ($T_H$ unavailable) are added together, newly generated secondary B cells are stimulated to AFC formation rather than inactivated.

Remarkably, although the presence of tolerogen prevents the generation of secondary B cells it does not preclude the subsequent generation of tertiarily responsive B cells. This finding led to the postulate that tertiary (quatenary, etc.) B cells are generated, not from secondary B cells, but rather from cells that are comparable to the progenitors in naive mice that give rise to secondary B cells. Although these cells would also be precursors of B cells (and therefore not themselves AFC precursors) and would be tolerance resistant, they would differ from naive precursors of memory B cells in that their Ig genes would be isotype switched and would have accumulated somatic mutations. Our studies now indicate that, in addition to secondary AFC precursors, such progenitors do exist in immunized mice. As anticipated, Ig genes of these cells are isotype switched and somatically mutated. We propose that these "memory progenitors of memory B cells" are the cells responsible for the propagation of the memory cell lineage and the progressive accumulation of somatic mutations.

**FZ 024** IMMUNOLOGICAL TOLERANCE WITHIN THE SECONDARY B LYMPHOCYTE REPERTOIRE, Gustav Nossal[1], Michael McHeyzer-Williams[2], Bali Pulendran[1], Michelle McLean[1] and Maria Karvelas[1], [1]The Walter and Eliza Hall Institute of Medical Research, Post Office, The Royal Melbourne Hospital, Victoria 3050, Australia, [2]Stanford University School of Medicine, Stanford, California 94305, USA.

When C57Bl/6 mice are immunised with $NP_{18}$ human serum albumin (HSA) adsorbed onto alum together with B pertussis adjuvant, a primary immune response ensues involving predominantly B cells using the VH 186.2 gene and the λ light chain. Also, cells are generated, presumably within the germinal centre, that display a higher affinity for NP than cells present within the primary B cell repertoire. Some of these higher affinity B cells also use VH 186.2 and λ light chains. An empirical trick can be used to enumerate these clonal precursors, through a repertoire analysis which focusses attention exclusively on IgG1 and a lightly-haptenated ELISA capture layer. With this technique, the number of clonable B cells, termed memory cells for short, rises from essentially 0 prior to immunisation, to around 100,000 at 14 days. The introduction of soluble, deaggregated, lightly haptenated NP-HSA before challenge immunisation virtually ablates the appearance of this new population. This can be observed through repertoire analysis, but equally through monitoring the progressive appearance of $NP^+$ $IgG1^+$ $\lambda^+$ $IgM^-$ cells with lymphocyte light-scattering characteristics by 6-parameter flow cytometry using the FACStar Plus. The VH genes of the memory cell population thus defined can be analysed by PCR analysis applied to single cells immediately ex vivo, using two rounds of amplification and nested primers. The analysis confirms a suprisingly rapid mutation and selection process beginning at about day 6 and reaching a high level of occurrence of the critical tryptophan to leucine substitution at position 33 in the CDR 1 region by day 12. Somewhat to our surprise, it is possible to turn the affinity maturation process of the germinal centre off not just by soluble antigen before immunisation, but even as late as day 6 after immunisation. This always works best when both hapten and carrier are used in the soluble deaggregated toleragen. However, carrier alone can reduce the memory cell numbers by a factor of 10, and hapten on an irrelevant carrier by a factor of 8, though not always as consistently. It thus appears that tolerance has been achieved within the secondary lymphoid repertoire through the deaggregated antigen affecting both T and B cells. It appears further that continued T cell help is required later in the germinal centre reaction than usually assumed. Recent results in which the germinal centre reaction is accelerated through carrier priming of host mice will be presented.

*Gene Targeted and Transgenic Animal Models*

**FZ 100 ANALYSIS OF LYMPHOCYTES IN *c-abl* MUTANT MICE.** Jeff Hardin[1], Sharon Boast[1], Pam Schwartzberg[1], Grace Lee[4], Fred Alt[1,2,4], Alan Stall[2], Elizabeth Robertson[3] and Stephen Goff[1,2]. Dept. of Biochemistry and Molecular Biophysics[1], Dept. of Microbiology[2], Dept. of Genetics and Development[3], and Howard Hughes Medical Institute[4], Columbia University, New York, NY 10032.

Mice homozygous for a targeted mutation in the c-*abl* tyrosine kinase have multiple abnormalities including high post-natal mortality, runting, and susceptibility to pulmonary infections. Analysis of the immune system of homozygous mutants using Fluorescence Activated Cell Sorting (FACS) analysis has shown significant but variable reductions of B and T lymphocyte populations, the most severely affected being B cell precursors in the bone marrow.

Further analysis of the B cell defect using four color FACS analysis demonstrates that pro-B cell fractions corresponding to distinct, developmentally coordinated stages of immunoglobulin locus expression and rearrangement are reduced by about half in mutants. This suggests that mutant bone marrow is affected from the earliest stages of B development, perhaps at the level of commitment of a multipotent progenitor to the B lineage. Further studies are underway using long term bone marrow cultures to elucidate this defect.

To assess the functionality of lymphocytes in c-abl mutants we have performed cytokine and mitogen stimulation assays. Colony forming unit (cfu)-Interleukin-7 (Il-7) assays of bone marrow is substantially decreased in mutants versus littermate controls. Similarly, mutant spleen and peripheral blood (PB) cfu-Lipopolysaccharide (LPS) is severely reduced. In addition, peripheral blood Concanavalin A stimulation is reduced. These data suggest that in addition to the deficit in number of various B lineage compartments, lymphocytes from mutant animals show functional defects in mitogen response assays. We are continuing our functional studies to determine if the decreased response is due to an intrinsic signaling defect in mutant lymphocytes due to the absence of the c-abl protein.

**FZ 101 GENERATION OF B-CELL DEFICIENT AND IG KAPPA DEFICIENT MICE BY TARGETED DELETION OF IG JH AND Jk/Ck LOCI.** Dennis Huszar[1], Mary Trounstine[1], Carole Kurahara[1], Fay Young[2], Chiung Chi Kuo[1], Yang Xu[2], Jeanne F. Loring[1], Frederick W. Alt[2], and Jianzhu Chen[2]; [1]GenPharm International Inc., 2375 Garcia Avenue, Mountain View, CA 94043, USA, and [2]The Howard Hughes Medical Institute and Children's Hospital, 300 Longwood Avenue, Boston, MA 02115.

We have generated an Ig kappa-deficient mouse strain and a B cell-deficient strain by gene targeting in embryonic stem cells. Kappa-deficient mice were generated by deletion of a 4.5 kb genomic fragment encompassing the Jk and Ck gene segments and the kappa intron enhancer. Mice heterozygous for the mutation have a two fold increase in the number of lambda$^+$ B cells relative to wild type littermates (~10% of the B cells were lambda$^+$ in heterozygotes compared to ~5% in control mice). In mice homozygous for the mutation all B cells expressed only lambda light chains, and the normal number of peripheral B lymphocytes was reduced by approximately 50%. No rearrangement of 3' kappa RS sequences was detected in the lambda$^+$ B cells of kappa-deficient mice.

Ig H chain deficient mice, generated by deletion of all four JH gene segments, completely lacked mature (sIg$^+$) B cells in the lymphoid organs. The B220$^+$/sIg$^-$ progenitor B cell population in the bone marrow was considerably reduced in number and consisted of immature B220$^+$/CD43$^+$ cells indicative of an early block in B cell differentiation, as expected. Deletion of the JH segments effectively silenced rearrangement of the H chain locus. No VH-D rearrangement was detected in either the bone marrow of homozygous mutant mice or in A-MuLV transformed fetal liver cells from mutant mice, nor were any aberrant rearrangements to sequences flanking the deleted JH region observed.

In addition to their utility in the study of B cell development and function, mice bearing null mutations of the Ig heavy and kappa light chain genes could enable the production of fully human antibodies from Ig knockout mice following introduction of germ line human Ig gene segments.

**FZ 102 TARGETED DELETION OF SWITCH REGION FLANKING SEQUENCES SHUTS DOWN IMMUNOGLOBULIN CLASS SWITCH RECOMBINATION,**

Steffen Jung and Andreas Radbruch
Institute for Genetics, University of Cologne, Cologne, FRG

Immunoglobulin class switch recombination has been shown to be a tightly regulated process. Upon instruction of activated B cells by cytokines, the actual recombinatorial event between $s\mu$ and a given downstream switch region is preceded by structural chromatin changes and transcriptional activation in the 5´ flanking sequences of the respective switch region. To determine role and importance of switch region flanking sequences for switch recombination, we generated mutant mice lacking 5´ flanking sequences of a particular switch region ($s\gamma1$). A 1.5 kb deletion of the 5´$s\gamma1$ flanking region renders the adjacent intact $s\gamma1$ region inaccessible for cytokine induced class switch recombination, thus identifying the region as recombination control element, spatially separated from the actual site of recombination.

With their $s\gamma1$ region shut down, homozygous 5´$s\gamma1\Delta$ mutant mice offer the possibility to analyse the importance of the frequently observed sequential class switch to IgE and the impact of the IgG1 deficiency on the development of the humoral response and memory formation.

**FZ 103 A TRANSGENIC MOUSE THAT EXPRESSES A DIVERSITY OF HUMAN SEQUENCE HEAVY AND LIGHT CHAIN IMMUNOGLOBULINS,** Nils Lonberg, Dennis Huszar, Lisa D. Taylor, Mary Trounstine, Stephen R. Schramm, Roshanak Mashayekh, Kay M. Higgins, Chiung-Chi Kuo, Clive Woodhouse, Robert M. Kay, and Condie E. Carmack, GenPharm International, 2375 Garcia Avenue, Mountain View, CA 94043.

We have generated transgenic mice that express a diverse repertoire of human sequence immunoglobulins. The expression of this repertoire is directed by light and heavy chain minilocus transgenes comprised of human protein coding sequences in an unrearranged, germ-line configuration. The heavy chain minilocus includes human $\mu$ and $\gamma1$ coding sequences together with their respective switch regions; both human heavy chain classes are expressed in animals that carry the transgene. In light chain transgenic animals the unrearranged minilocus sequences recombine to form VJ joints that use all five human $J_K$ segments, resulting in a diversity of human-like CDR3 regions. Similarly, in heavy chain transgenics the inserted sequences undergo VDJ joining complete with N region addition to generate a human-like $V_H$ CDR3 repertoire. All six human $J_H$ segments and at least eight of the ten transgene encoded human D segments are expressed. When we breed these transgenic animals into a background in which the endogenous heavy chain locus has been inactivated by homologous recombination in embryonic stem cells, we find that the human minilocus is able to rescue the B-cell lineage. These mice represent a potential source of human sequence antibodies for *in vivo* therapeutic applications.

FZ 104 IMMUNOGLOBULIN D DEFICIENT MICE CAN MOUNT NORMAL IMMUNE RESONSES,
Lars Nitschke, Georges Köhler and Marinus C.Lamers, Max-Planck-Institut für Immunbiologie, 78 Freiburg, Germany

To examine the in vivo function of IgD we generated mice deficient for IgD by gene targeting. The IgD$^-$ mice show a reduced B-cell compartment with 30 to 50 % less B cells in the spleen and lymph nodes. The pre-B-cell compartment is not affected as judged by the number and phenotype of B-lineage cells in the bone marrow. The sIgD$^-$ B cells express 2-3 times more sIgM than B cells of control animals. The serum concentrations of the various Ig isotypes of IgD$^-$ mice are almost normal, indicating that surface IgD expression is not necessary for class switching of B cells. Immunisation experiments showed that IgD$^-$ mice were able to respond well to thymus-dependent and -independent antigens. After immunisation normal germinal centers developed in the IgD$^-$ mice. These data suggest that IgD is not necessary for the induction of immune responses, but may play an important role in the homeostasis of cells in the B-cell compartment. Further experiments studying the specific role of IgD will be presented.

FZ 105 **Increased levels of expression of an IgH transgene potentiated by the IgH intron and IgH 3´enhancers in transgenic mice.**
Sven Pettersson (1) Velmurugesan Arulampalam (1), Annika Samuelsson (1), Urban Lendahl (2) and Inger Lundkvist. (3) Center For Biotechnology (1), and Center For Molecular Biology (2), Karolinska Institute, and Dept of Clinical Immunology, Huddinge (3), Sweden

The expression of immunoglobulin genes is subject to allelic exclusion. We as well as others have reported that whilst an Ig-transgene does indeed exert a considerable inhibition of rearrangement of the endogenous loci, this inhibition is extremely leaky and these transgenic animals still mantain a large population of B cells expressing endogenous rearranged immunoglobulins. To further our understanding on the mechanisms of allelic eclusion and the reasons behind the "leakyness" of the endogenous Ig-loci, transgenic constructs with or without the distal IgH 3´enhancer were made. The transgenic Ig-gene potentiated by both the IgH intron enhancer and the IgH 3´enhancer revealed increased expression level of the transgene as compared to Ig-construct only potentiated by the IgH intron enhancer. The presence of the IgH 3´enhancer does not prevent the increase of numbers of cells expressing endogenous Ig-genes. The pattern of expression in various lymphoid organs and the effect following antigenic activation of transgenic spleen cells will be presented. The IgH 3´enhancer also confers lymphoid restricted expression of a reporter in transgenic mice and data will be presented regarding the temporal and spatial activation of this enhancer

FZ 106 IMMUNOGLOBULIN D-DEFICIENT MOUSE,
Jürgen Roes and Klaus Rajewsky, Institute for Genetics, University of Köln, Weyertal 121, D-5000 Köln 41, FRG

To assess the role of IgD *in vivo* we generated IgD-deficient mice by gene-targeting and studied B cell development and function in the absence of IgD expression. In the mutant animals, conventional and CD5 positive (B1) B cells are present in normal numbers and the expression of the surface markers CD22 and CD23 in the compartment of conventional B cells indicates aquisition of a mature phenotype. As in wild type animals, most of the peripheral B cells are resting cells. The IgD-deficient mice repond well to T cell independent and dependent antigens. However, in heterozygous mutant animals B cells expressing the wild type IgH locus are overrepresented in the peripheral B cell pool and T cell dependent IgG1 responses are further dominated by B cells expressing the wildtype IgH locus. Similarly, in homozygous mutant (IgD-deficient) animals affinity maturation is delayed in the early primary response compared to control animals, although the mutants are capable of generating high affinity B cell memory. Thus, rather than being involved in major regulatory processes as had been suggested, IgD seems to function as an antigen receptor optimized for efficient recruitment of B cells into antigen driven reponses. The IgD-mediated acceleration of affinity maturation in the early phase of the T cell dependent primary response may confer to the animal a critical advantage in the defense against pathogens. Data obtained using IgD-deficient mice as a model system will be presented.

FZ 107 REGULATION OF B CELLS IN MICE TRANSGENIC FOR IMMUNOGLOBULIN GENES ENCODING A DISEASE-ASSOCIATED ANTI-SELF-IgG2a (RHEUMATOID FACTOR)
*+Mark J. Shlomchik, *Dorit Zharhy, #Sally Camper and *Martin G. Weigert *Fox Chase Cancer Center,+Warner-Lambert Life Sciences Research Foundation Fellow, and #University of Michigan

"Tolerance", the antigen-specific process by which the normal immune system maintains unresponsiveness to self, has recently been shown to directly affect B cells (Nemazee, et. al. and Goodnow, et. al). Given the potential effects of "tolerance" directly on B cells, it is reasonable to hypothesize that autoantibodies occur in autoimmune disease because of defects in the normal tolerance mechanisms which otherwise would prevent activation of self-specific B cells by antigen. However, a problem in such a model is how to account for the unique specificities of autoantibodies in autoimmune disease. For example, anti-dsDNA is a hallmark of SLE while anti-IgG (rheumatoid factor [RF]) occurs in rheumatoid arthritis. As an alternative to the aforementioned hypothesis, it is possible that tolerance occurs for B cells of only some specificities and that this explains why certain types of autoantibodies dominate in certain autoimmune diseases.

To address this issue, we have established a rheumatoid factor transgenic model of autoimmunity, using V genes derived from an IgA anti-IgG2a RF isolated from an autoimmune MRL/lpr mouse. The RF we chose binds only IgG2a$^a$ allotype but not IgG2a$^b$. We have produced IgH congenic transgenic animals which either express or lack the self-antigen. Using mice which lack the self-antigen, we show by FACS and hybridoma analysis that the transgene is expressed to the exclusion of endogenous genes in most splenic B cells. The frequency and numbers of transgene-expressing B cells are similar in peripheral lymphoid organs of mice which have the self-antigen and those which lack it. Thus, B cells expressing an anti-self IgG2a surface receptor can develop in this system. We are currently assessing whether such B cells are fully functional. In this regard, studies of spontaneous serum expression of the transgene-encoded IgM reveal two interesting features: 1) the transgene IgM comprises the minority (usually less than 3%) of the total IgM in mice which lack the autoantigen and 2) there is significantly more Tg-encoded IgM in mice which have the autoantigen, although it still averages only about 10% of the total serum IgM. The implications of these serum data are unclear at present; possible explanations will be discussed.

**FZ 108** GENERATION OF A $V_H$ TRANSGENIC MOUSE BY GENE TARGETING, Shinsuke Taki and Klaus Rajewsky, Institute for Gentics, University of Cologne, D-5000 Cologne, FRG

A rearranged $V_HT15$ gene was inserted into the germline $J_H$ locus of embryonic stem cells by gene targeting and simultaneously all four $J_H$ genes were deleted. Mutant mice having this mutation on both chromosomes express $V_HT15$ positive IgM in the serum and on the cell surface of splenic B cells. All IgG subclasses and IgA serum antibodies were observed in the serum indicating that the inserted $V_HT15$ gene underwent class switching to downstream C-genes. One would expect that an already rearranged, functional VDJ segment on one chromosome inhibited the other chromosome to undergo V to DJ rearrangement. Surprisingly, however, more than half of B cells in heterozygous mutant mice express IgM molecules of the allotype of the wildtype allele. Allelic exclusion appears to work properly as shown by the absence of double producers. Analysis of the targeted allele in wildtype IgM-expressing B cells showed that a secondary rearrangement, possibly mediated by an upstream D element and the internal heptamer which resides in the $V_H$ gene, destroyed the $V_HT15$ gene. Even in homozygous mutant mice, we found some B cells which were not stained with an anti-$V_HT15$ antibody in the peritoneal cavity but not in the peripheral blood or in the spleen. This observation may indicate that peritoneal B cells have a different activity for V gene rearrangement although it could also be due to selection of rare B cells in the peritoneal cavity. Regardless of this apparent leakiness, serum IgM of homozygous mice bear $V_HT15$ almost exclusively and, thus, this mutant mouse should be useful to analyze post-immunization events such as cellular recruitment, somatic mutation and class switching.

**FZ 109** THE CD5/Ly-1 DEFICIENT HOMOZYGOUS MICE, Alexander Tarakhovsky, Werner Müller, Klaus Rajewsky, Department of Immunology, Institute for Genetics, University of Cologne, Cologne 41 D-5000, FRG

The CD5 antigen is expressed on the surface of T cells and additionaly marks a distinct minor subpopulation of B cells, the so called CD5 (Ly-1) or B1a lymphocytes. The ability of CD5 to interact with the pan-B lymphocyte surface antigen CD72/Lyb-2 suggests the involvement of CD5/Ly-1 in cognate T/B cell interaction and also in self-renewal of B1a cells.

To analyse the physiological role of the CD5 protein in lymphocyte function, we inactivated the CD5 gene by homologous recombination. The exon VII encoding the transmembrane domain of the CD5 protein has been replaced by the *neo* gene and a frameshift mutation was introduced in exon III, which prevents the expression of the potentially functional soluble CD5 extracellular domain.

As expected, mice homozygous for the disrupted allele of the CD5 gene do not express the CD5 antigen on the surface of T cells and peritoneal B1 cells.

The data on B and T cell development as well as an analysis of T cell-dependent and -independent immune responses in CD5-deficient homozygous mice will be presented.

**FZ 110** ROLE OF B CELLS IN T- CELL PRIMING IN VIVO

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In order to investigate the participation of B cells in T cell priming we used two different experimental models :the μM-targeted C57Bl.6 mice and the Ac38 transgenic C57Bl.6 mice.

After local injection of the antigen (Keyhole Limpet Hemocyanin .KLH) the T- cell responsiveness in draining lymph nodes and spleen, was assayed by proliferation analysis and lymphokine production (inter--leukins 2,3 and 5 and gamma-interferon) production after mitogenic and antigenic re-stimulation in vitro.

The results showed that,in both experimental models,T cells from lymph nodes origin were not primed for KLH while a slight, but significant priming was observed with splenic T lymphocytes.

A comparative analysis of the antigen presenting cells (macrophages and dendritic cells) in spleen and lymph nodes of targeted and transgenic animals is presently underway.

These models of in vivo T- cell priming in B cell deficient mice and one-specificity bearing B cells mice led us to propose thd the antigen presenting cell function of B cells to T cells in vivo might be more important that presentation by macrophages and dendritic cells.

**FZ 111** GENERATION OF A MOUSE' STRAIN CARRYING HUMAN Cκ GENE AT ITS GERMLINE LOCUS, Yongrui Zou and Klaus Rajewsky,Institute of Genetics, University of Cologne,5000 Cologne 41, FRG

It has been shown that human antibodies are highly efficient for immunotherapy in man, with significant less immunogenicity than rodent antibodies. However, the sources by which human antibodies are obtained, in practice, are limited. One of the alternate technologies, therefore, is to develop humanized rodent antibodies through the fusion of mouse IgV regions with the C regions of human Ig.

We are now trying to develop a mouse strain which, after immunization, can directly produce chimeric antibodies carrying the mouse IgV domains attached to the C domains of human κ and γ1 chains. The strategy we have developed is to replace the germline C region genes of mouse Igκ and $IgG_1$ chains with the corresponding human genes by gene targeting. The homozygous mice that carry human Cκ at the position of mouse Cκ gene locus were obtained. The B cells that generated in this mutant mice can be stained with anti-human Igκ antibodies, but not with antibodies against mouse Igκ. In sera, around 2 mg/ml of chimeric Igκ can be detected. After immunization with antigens, the mutant mice can mount immune responses equivalent to normal mice.

We are now still in the process to make the mouse strain that carry human $IgG_1$ C region gene in the position where mouse $IgG_1$ C region gene located.

*B Cell Differentiation Steps & Pathways*

**FZ 112** RESOLUTION AND FUNCTIONAL ANALYSIS OF NEWLY EMERGING PERIPHERAL B CELLS IN ADULTS, David M. Allman, Stacy E. Ferguson, Vicky M. Lentz and Michael P. Cancro, Dept. of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Phila., PA 19104

Although recently generated B cells mature further following egress from the marrow, this pivitol stage of development is poorly characterized. We have identified an adult splenic B cell subpopulation defined by high surface expression of heat-stable antigen ($HSA^{hi}$). $HSA^{hi}$ B cells predominate both in early life and during the initial stages of reconstitution in either marrow chimeras or sublethally irradiated adults. Further, $HSA^{hi}$ B cells differentiate to a $HSA^{lo}$ phenotype within 4 days of transfer to C.B17*scid* mice. These results indicate that $HSA^{hi}$ B cells are the least mature B cells in the spleen, and that this differentiation pathway reflects the origin of some, if not all, mature peripheral B cells. Furthermore, $HSA^{hi}$ B cells exhibit functional characteristics considered hallmarks of immaturity: They are refractory to sIgM crosslinking but respond to LPS; and can respond to antigen in the presence of primed $T_H$. Finally, adoptively transferred $HSA^{hi}$ B cells can reconstitute both primary and memory humoral responses.

**FZ 113** B LYMPHOCYTE DEVELOPMENT IN TRANSGENIC MICE EXPRESSING AN ANTI-PHOSPHATIDYL CHOLINE SPECIFIC, REARRANGED VH12 GENE. Larry W. Arnold, Christopher A. Pennell*, Suzanne K. McCray, and Stephen H. Clarke. Department of Microbiology and Immunology, Univ. of North Carolina, Chapel Hill, NC 27599 and * Department of Laboratory Medicine and Pathology, Univ. of Minnesota, Minneapolis MN 55455. The most frequent naturally occuring B cells express an immunoglobulin (Ig) specific for phosphatidyl choline (PtC) and belong exclusively to the B1 type (formerly CD5 B cells). This Ig is encoded predominantly by 2 VH-VL gene pairs; VH11/Vκ9 and VH12/Vκ4. Many of the details of B1 cell development are unknown, particularly, the basis of the striking differences in Ig repertoire between these cells and the predominant B cell in adult spleen (currently called B2). To investigate the details of the development of B cells expressing IgM specific for PtC we have made transgenic (Tg) mice expressing either the rearranged VH12 gene or Vκ4 gene of CH27, a $CD5^+$ B cell lymphoma whose IgM is specific for PtC. Two lines each of VH12 and Vκ4 Tg mice have been established. Young adult mice of the 7-2 VH12 line express B1 cells that are predominantly anti-PtC whereas the majority of B2 cells express endogenous heavy chains. 6-1 VH12 line adult mice exclude the use of endogenous heavy chain rearrangements and have B1 cells that are nearly all anti-PtC. 6-1 line Tg mice have $Tg^+$ splenic B cells which appear to be B2 cells in that they are $CD5^-$ and $CD23^{++}$ but are not specific for PtC although the total number of B2 cells is reduced. Newborn mice of both lines have newly emerging B cells which are nearly all $Tg^+$ but almost none are specific for PtC. Introduction of the *xid* mutation into the 6-1 VH12 Tg mice results in drastically reduced numbers of B1 cells consistent with the observed defect in this B cell subset in these mice. However, we find that B2 cells are also drastically reduced in line 6-1/*xid* mice suggesting that the xid defect affects more than just B1 cells. Mice with both VH12 and Vκ4 Tgs appear to produce only anti-PtC B cells but both B1 and B2 cells are present. The mice with both Tgs have about 4 times normal numbers of splenic B cells as well as T cells. These data demonstrate that the VH12 gene, as well as the anti-PtC specificity, can be expressed by conventional B cells. They also provide additional evidence that, among B1 cells, the PtC specificity is selected from a larger repertoire by antigen-driven clonal selection. The functional characteristics of the B1 and B2 cells are under investigation.

**FZ 114** HETEROGENEITY OF CHRONIC LYMPHOCYTIC LEUKEMIA: PHENOTYPIC AND FLUORESCENT *IN SITU* HYBRIDIZATION STUDIES, Douglas C. Aziz, M.D., Ph.D. and Shaokee Wu, Ph.D., OncQuest, Division of Specialty Laboratories, Inc., 3520 Dunhill St., San Diego, CA 92121

Chronic lymphocytic leukemia (CLL) is distinguished from the more aggressive prolymphocytic leukemia (PLL) by the presence of weak surface immunoglobulin expression and the co-expression of CD5 along with the B-cell antigens (CD19, CD20, HLA-DR). However, some cases of PLL co-express CD5 as well. Trisomy 12 is the most common cytogenetic abnormality found in CLL and is associated with an increased need for early treatment. In order to characterize the lineage heterogeneity in the chronic B-cell leukemias, the intensity of the surface marker expression was determined by flow cytometry and numerical aberrations of chromosome 12 were determined by fluorescent *in situ* hybridization. The immunophenotype of CLL consisted of weak monoclonal surface immunoglobulin expression and intense CD19, CD5 and HLA-DR expression. CD20 expression was heterogeneous, ranging from weak expression (mean peak intensity fluorescence (MPIF) = $70\pm21$) in 25 of 40 cases (63%), to moderate expression (MPIF = $159\pm26$) in 8 of 40 cases (20%), to high expression (MPIF = $427\pm183$) in 6 of 40 cases (15%). One case of CLL did not express CD5. Trisomy 12 was observed in 2 cases, one case of CLL had intense expression of CD20, (MPIF = 550) and the other CLL had weak expression of CD20, (MPIF = 35). PLL, as defined by intense monoclonal surface immunoglobulin expression, consisted of four subtypes depending on the intensity of CD20 and CD5 expression. In the CD5 negative group, 11 of 18 (61%) had intense expression of CD20 (MPIF = $981\pm629$) and 3 of 18 (17%) had moderate expression of CD20 (MPIF = $180\pm28$). In the cases that expressed CD5, 3 of 18 (17%) had intense expression of CD20 (MPIF = $697\pm271$) and 1 of 18 (6%) had weak expression of CD20 (MIPF = 120). Expression of CD20 is heterogeneous in chronic lymphocytic and prolymphocytic leukemia, and the clinical significance of these variant forms needs to be determined.

**FZ 115** **IDENTIFICATION OF AN ENDOGENOUS ANTIGEN IN A CD5+ B CELL TUMOR LINE: EVIDENCE FOR TUMORIGENESIS MEDIATED BY ANTIGEN RECEPTOR** $^+$Gabriele Beck-Engeser, $^+$Ulrike Keyna, $^o$Steven Applequist, $^+$Klaus Bornemann, and $^{+,o}$Hans-Martin Jäck, $^+$Department of Microbiology and Immunology and $^o$Program in Molecular Biology, Loyola University of Chicago, Maywood, IL 60153

There is a strong selection for a particular V region in B cell tumors in B/W mice, the conventional designation for (NZB x NZW)F1 hybrids. Based on this observation data, it was suggested that these tumors are formed during the clonal expansion that follows antigenic stimulation. From a tumor that originated in a BW mouse, we adapted the $CD5^+$ and surface IgM positive B lymphoma line, NYC. The heavy chain variable region of IgM expressed by NYC tumor cells is almost identical to that of other, independent B cell tumors found in BW mice. NYC tumor cells lose the capacity to grow in culture when they do not synthesize surface IgM. IgM produced by NYC tumor cells binds a viral antigen synthesized by the same tumor cells and, unlike transfected immunoglobulin with a different variable region, NYC-IgM is concentrated in intracellular vesicles that presumably contain viral precursor proteins. When NYC tumor cells are fused with a plasmacytoma, the vesicles are much larger and resemble Russell bodies under the fluorescence microscope. In electron photomicrographs, they show more fine structure than do conventional Russell bodies and resemble myelin figures. We suggest that the interaction of an endogenous antigen with surface IgM continuously stimulates growth of B cells and, thus, that the tumorigenesis of B lymphomas in BW mice is mediated by antigen receptors.

FZ 116 CHARACTERIZATION OF B220$^+$ AND B220$^-$ THYMUS TARGETS FOR ABELSON MURINE LEUKEMIA VIRUS, Eric Y. Chen[1], Bradley J. Swanson[2], and Steven S. Clark[1,2], Department of Human Oncology[1], Cellular and Molecular Biology Program[2], University of Wisconsin, Madison, WI 53792.

B220$^+$ transformed lymphocytes are a significant component in primary murine thymic lymphomas induced by the v-*abl* expressing Abelson murine leukemia virus (A-MuLV). Since B220$^+$ cells are exceedingly rare in normal thymi, the nature and significance of v-*abl* transformed B220$^+$ cells are unclear. The B220$^+$ thymic lymphocytes may represent pre-B cell transformants since others have established B220$^+$, Thy-1$^-$ pre-B lymphocyte cell lines from v-*abl* and v-*myc* transformed thymi. Alternatively, these B220$^+$ transformants may arise from a thymic B220$^+$, Thy-1$^{lo}$ lymphocyte progenitor transformation target. A progenitor with a similar phenotype is found in the bone marrow and is a highly preferred target for A-MuLV transformation. In order to clarify the nature of the v-*abl* transformed B220$^+$ thymic lymphocytes, the cells from A-MuLV primary thymomas were separated into B220$^+$ and B220$^-$ groups for phenotype analysis. The data indicate that the B220$^+$ targets express Thy1$^{lo}$ and resemble the bone marrow lymphocyte progenitor. The B220$^-$ cells are Thy1$^{hi}$ T-lymphocytes that are heterogeneous for CD3, CD4, and CD8. DNA blot analysis is underway to further characterize the B220$^+$ transformants and the relationship between the B220$^+$ and B220$^-$ populations.

FZ 117 LYMPHOCYTE TRANSFORMATION TARGETS FOR *BCR-ABL* AND V-*ABL* ONCOGENES. S. S. Clark, E. Chen, M. Fizzotti, A. Krueger. Dept. of Human Oncology, Univ. of Wisconsin, Madison, WI

The Philadelphia chromosome translocation (Ph) appears in hematopoietic precursor cells and encodes chimeric P210 and P185 *BCR-ABL* oncogenes. Consequently, the *BCR-ABL* tyrosine-specific kinases can be expressed in most hematopoietic lineages and affect the pathogenesis of multiple human leukemias. One exception is that even when the Ph chromosome and *BCR-ABL* expression is found in T lymphocytes or in multipotential progenitors, T lymphoid malignancy is rare. In contrast, B lymphocytes are readily transformed by *BCR-ABL*. To investigate the ability of *BCR-ABL* oncogenes to transform T lymphocytes, retroviruses engineered to express P210 or P185 were injected into the thymi of young mice. The data show that *BCR-ABL* oncogenes efficiently induced clonal thymic lymphoma involving immature thymic lymphocytes. Compared to Moloney and Abelson murine leukemia viruses, *BCR-ABL* viruses transformed distinct subsets of thymic lymphocytes. As expected, Mo-MuLV thymomas consisted of Thy-1$^+$ T cells, while v-*abl* and *BCR-ABL* thymic lymphomas often consisted of a mixed B220$^+$, Thy-1$^+$ population. Several *BCR-ABL*- but not v-*abl*-induced tumors expressed carboxy-terminal truncated oncoproteins. The data demonstrate a unique interaction between *BCR-ABL* kinases and immature T lymphocytes which is not seen between *BCR-ABL* and other hematolymphoid cell types, or between v-*abl* and T lymphocytes. Furthermore, exceedingly rare B220$^+$ lymphocytes in the thymus are highly preferred v-*abl* and *BCR-ABL* transformation targets.

FZ 118 CARACTERIZATION OF B-CELL PRECURSORS DETECTED IN THE YOLK SAC AND IN THE EMBRYO BODY OF EMBRYOS AT DAY 9 OF GESTATION. Ana Cumano, Caren Furlonger and Christopher J. Paige. Unité de Biologie Moléculaire du Gène, Institut Pasteur, Paris, France and The Ontario Cancer Institute, Toronto, Canada.

The first hematopoietic precursors have been detected in the yolk sac of embryos at day 9 of gestation. We isolated cells from yolk sac and from the embryo body of embryos at day 9 and 10 of gestation and selected cells by the expression of two cell surface markers present in long term reconstituting hematopoietic stem cells: AA4.1 and Sca-1/Ly6A. B220 positive cells are detected after 10 days of "in vitro" culture in the AA4.1 but not in the Ly6A/Sca-1 positive precursors. Those cells can further differentiate to immunoglobulin secreting cells upon mitogen stimulation. B-cell precursors detected under those conditions are capable of extensive cell division at the stage of uncommitment for immunoglobulin heavy chain expression and can generate from a single precursor mature B cells that express both V gene families that lie 5' and 3' of the S107 family. Cells acquire the capacity to respond to LPS two days later than a macrophage/B-cell bipotent precursor detected in fetal liver at day 12 of gestation. This observation together with the extensive proliferation capacity sugest that this precursor cell is a more immature cell than the macrophage/B-cell precursor.

FZ 119 B CELL DIFFERENTIATION IN THE RAT: THY-1 EXPRESSION ON NEWLY FORMED B CELLS IN RAT PERIPHERAL LYMPHOID ORGANS. Nynke K. de Boer, Gerrit Jan Deenen, Tjitske de Boer, Aaron B. Kantor* and Frans G.M. Kroese. *Dept. Histology and Cell Biology, University of Groningen, Groningen, The Netherlands and *Dept. Genetics, Stanford University Medical School, Stanford, CA, USA.*

In the bone marrow many newly formed B cells (NF-B cells) are produced every day, whereas only relatively few are incorporated in the pool of mature peripheral B cells. This recruitment is based upon positive and negative selection mechanisms. In rats we are able to distinguish NF-B cells in BM, PBL and spleen (but not in LN, PP and TDL). They characteristically express high levels of sIgM and HIS24-determinant (anti CD45R), but low levels of sIgD (IgM$^{br}$IgD$^{du}$HIS24$^{br}$). This contrasts the phenotype of the vast majority of mature B cells which are IgM$^{du}$IgD$^{br}$HIS24$^{br}$ (small follicular B cells) or IgM$^{br}$IgD$^{du}$HIS24$^{du}$ (marginal zone B cells). Here we further define sIgM$^+$ B cells in rats by three and four color flow cytometry. We have shown that Thy-1 is expressed at high levels by all NF-B cells and a subpopulation (approx. 5%) of the small follicular B cells in LN and spleen. Cell kinetics studies using long-term BrdU labelling show that both Thy-1$^+$ B cell subsets have a high turnover rate (25-50% per day), whereas Thy-1$^-$ peripheral B cells turnover with a rate of approximately 1% per day. Furthermore, following the development of B cells in neonates and in lethally irradiated rats, reconstituted with bone marrow, we observed that the first sIgM$^+$ B cells that develop are the Thy-1$^+$ NF-B cells. Thy-1$^+$ follicular B cells appear later, followed by Thy-1$^-$ follicular B cells and finally marginal zone B cells. The appearence of these B cell subsets presumabbly represents sequential stages of normal B cell development in rats. The cell kinetics data demonstrate that a major cell loss occurs at the transition between Thy-1$^+$ and Thy-1$^-$ cells. This process is very likely associated with selection phenomena.

FZ 120 *Pim*-1 EXPRESSION LEVELS DETERMINE GROWTH FACTOR RESPONSES OF EARLY B-CELLS IN BONE MARROW, Jos Domen, Nathalie van der Lugt, Dennis Acton and Anton Berns, Division of Molecular Genetics, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

The mouse proto-oncogene *Pim*-1 encodes two cytoplasmic serine/threonine specific protein kinases. The highest expression levels are seen in haematopoieic cells and organs like thymus, spleen and fetal liver, but also in gonads and ES cells. Expression can be induced by growth factors like IL-2 and IL-3. *Pim*-1 has been found activated (overexpressed) by retroviral insertion in haematopoietic tumors. E$\mu$-*Pim*-1 transgenic mice overexpressing the gene are very susceptible to the induction of lymphomas (both spontaneous or MoMuLV or ENU induced), without an obvious prenoplastic state being apperant. Flow cytometric analysis of *Pim*-1 null mutant mice reveals no obvious abnormalities in their lymphoid compartments. However, some growth factor responses are abnormal in these mice, e.g. the proliferative response to IL-3 of bone-marrow derived mast-cells is impaired. When we analyzed the early B cell compartment in these mice we found serious impairments in the response to the growth factors IL-7 and SF (steel factor), both in colony assays and direct liquid cultures of bone marrow. This phenotype is intermediate in heterozygotes and could be rescued by crossing in a wild-type *Pim*-1 transgene in the null mutant mice. Analysis of mice overexpressing *Pim*-1 from an activated transgene (E$\mu$-*Pim*-1) revealed an increased response to these growth factors, which was stronger in mice homozygous for this transgene than in heterozygous mice, again revealing a dosage effect. We also found that Whitlock-Witte type long term cultures from bone marrow of E$\mu$-*Pim*-1 transgenic but not of wild-type mice readily yielded factor dependant cell lines with a primitive lymphoid phenotype (B220$^+$, sIg$^-$, CD4$^+$). The implications of this for the functioning of *Pim*-1 in the early lymphoid compartments will be discussed.

FZ 121 REGENERATION OF CD5$^+$ AND CD5$^-$ B1 B CELLS FROM MOUSE ADULT BONE MARROW CELLS, Elliott Meenal, Department of Microbiology and Division of Developmental and Clinical Immunology, University of Alabama at Birmingham, Birmingham, AL 35294

In newborn SCID recipients of BALB/c adult bone marrow depleted of B220$^+$ and CD5$^+$ cells, normal numbers of donor derived B1 B cells that expressed IgM, CD11b, IL5R and low levels of IgD were detected in the peritoneal lavage 14 to 20 weeks after transplantation. 15 to 20 % of the IgM$^+$ cells also expressed CD5. In recipients of whole marrow cells, a gradual decline in the capacity to generate CD5$^+$ B1 B cells was noted with increase in donor age from 4 to 40 weeks. SCID mice up to 21 days of age were able to support the generation of donor derived CD5$^+$ and CD5$^-$ B1 B cells. In irradiated adult SCID recipients, the majority of donor derived B cells consisted of IgM$^+$, IgD$^{lo}$, CD23$^+$, conventional B cells. Regeneration of B1 B cells was poor and CD5$^+$ B cells were absent. Treatment of adult recipients with anti-asialoGM1 antisera increased the numbers of B1 B cells and restored small but significant numbers of CD5$^+$ B cells.

In lethally irradiated adult C57Bl/6 recipients of whole marrow cells from C57Bl/6.IgH$^a$ congenic donors, treatment with anti-asialoAGM1 antisera or monoclonal anti-NK1.1 greatly enhanced generation of donor derived B1 B cells over that in untreated recipients. In contrast, monoclonal anti-IFN-$\gamma$ ablated B1 B cells almost entirely. CD5$^+$ B cells remained absent in all groups of C57Bl/6 recipients, perhaps, reflecting influences of other components of the residual immune system. In summary, both CD5$^+$ and CD5$^-$ B1 B cells can be restored from adult bone marrow in SCID recipients. The age of recipient rather than donor affects regeneration of B1 B cells, which is further influenced by the presence of NK cells, IFN-$\gamma$, and other lymphocyte derived factors which persist following lethal irradiation. Supported by NIH grants AI23694 and AI30879.

FZ 122 ANALYSIS OF B CELL SUBSETS DEFINED BY THE J11D MARKER: SURFACE J11D DENSITY IS INVERSELY CORRELATED WITH B CELL MATURITY, Stacy E. Ferguson*#, David M. Allman * and Michael P. Cancro, *University of Pennsylvania, School of Medicine, Immunobiology Division, Philadelphia, PA, #Present address: University of Michigan School of Medicine, Ann Arbor, MI

The function of the surface marker, J11D (or Heat Stable Antigen), has not been determined. J11D hi and J11D lo B cell subsets have been suggested to represent distinct B cell lineages, responsible for primary and secondary antibody responses, respectively. Here, we show that J11D hi B cells are immature with respect to expression of other B cell markers (sIgD, B220 and Class II MHC). These cells comprise the majority of neonatal B cells but less than 10% of adult splenic B lymphocytes. Adult J11D hi cells proliferate in the presence of bacterial lipopolysaccharide but do not respond to treatment with anti-IgM or PMA plus Ionomycin. We suggest that J11D hi and J11D lo subsets do not represent distinct lineages, but that J11D hi cells are the progenitors of the J11D lo population. These populations exhibit multiple phenotypic and signaling characteristics suggestive that immature B cells are J11D hi.

FZ 123 REGULATION OF bcl2 GENE EXPRESSION DURING B LYMPHOCYTE DIFFERENTIATION, Urszula Hibner, Matthias Haury, Antonio Freitas, Véronique Hermitte and Antonio Coutinho, Unité d'Immunobiologie, Institut Pasteur, 25 rue du Docteur Roux, 75724Paris Cedex 15, France.

In a functional immune system there is a continuous production of B lymphocytes in the bone marrow followed by their migration into the periphery. The size of the B lymphocyte population is determined both by the production of precursors and by elimination of cells. It appears that the elimination, through a process of apoptosis, is, at least in part, a function of a cell's V-region reactivity.

The product of bcl2 gene has been shown to protect lymphocytes from several inducers of apoptosis. We have used the RNAse protection assay in an attempt to correlate the expression of this gene with successive stages of murine B lymphocyte differentiation.

The expression of the endogenous bcl2 gene is low in B lymphocyte precursors in the bone marrow and it increases progressively as the cells differentiate into bone marrow B, peripheral B and long-lived peripheral B lymphocytes.

Since these B cell populations display different V-region repertoires, they must have undergone selection. It appears therefore that the increased bcl2 gene expression correlates with successive passages through selective steps.

Interestingly, the highly selected peritoneal cavity B lymphocytes have a low level of bcl2 gene expression, possibly due to their seemingly activated phenotype. Indeed, we have observed that the levels of bcl2 transcripts in resting splenic B cells are decreased upon activation *in vitro*. However the *in vivo* activated splenic B lymphocytes retain high levels of the bcl2 gene expression.

**FZ 124** IgA PLASMA CELLS IN μ,k TRANSGENIC MICE BELONG TO THE B-1 (CD5 B) CELL LINEAGE. Frans G.M. Kroese, Willem A.M. Ammerlaan and Aaron B. Kantor. *Dept. Histology and Cell Biology, Immunology Section, Univ. Groningen, The Netherlands and Dept. Genetics, Stanford Univ. Med. School, Stanford, CA.*

B6-Sp6 transgenic mice carry fully rearranged (Balb/c-derived, Igh-$C^a$ allotype) μ heavy chain and k light chain transgenes, specific for TNP, on a C57Bl background (Igh-$C^b$ allotype). FACS analyses show that the majority of B cells in peripheral lymphoid organs and BM express transgenic IgM exclusively. A small proportion of the B cells, however, express endogenous IgM, usually concomitant with transgenic IgM. Three criteria establish that the large majority of the endogenous IgM expressing B cells belong to the B-1 cell lineage. First, endogenous IgM expressing B cells in B6-Sp6 mice have the same localization pattern as B-1 cells from normal animals: they are enriched in the peritoneal cavity. Second, the vast majority of the endogenous $IgM^+$ B cells have the phenotype of B-1 cells: the endogenous $IgM^+$ peritoneal B cells express Mac-1 and low levels of IgD and most also express CD5 (Ly-1). Third, B6-Sp6 BM poorly reconstitutes endogenous $IgM^+$ B cells, just as adult BM from normal mice poorly reconstitutes B-1 cells. In contrast, B cells which only express the transgene are readily reconstituted by B6-Sp6 BM. The few endogenous $IgM^+$ cells in the B6-Sp6 BM recipients are located in the peritoneal cavity and have the phenotype of B-1b cells (previously the Ly-1 B sister population), which are known to be reconstituted by adult BM. Two color immunofluorescence staining from isolated gut lamina propria cells shows the presence of many IgA containing cells, about one-third of which simultaneously express cytoplasmic (transgenic) IgM. This IgA can only be derived from endogenous gene expressing cells in these transgenic mice. Since almost all endogenous $IgM^+$ cells are B-1 cells (both B-1a and B-1b), the data strongly indicate that the intestinal IgA plasma cells also belong to the B-1 cell lineage.

**FZ 125** ***In Situ* Hybridization Detection of μ mRNA in Normal Pre - B Cells and B Lymphocytes from Rat Bone Marrow and Spleen**

**Y.H.Kwong and D. Opstelten, Dept. of Biochemistry, University of Hong Kong, Hong Kong**

This work is supported by grant no. 338/032/0003 from the Research Grants Council, Hong Kong

For the study of B lymphocyte development in the bone marrow (BM) microenvironment of rat and mouse, we aim to develop highly sensitive techniques allowing simultaneous detection of protein and mRNA by combined immuno- and hybridohistology. Here we report the results of two non-radioactive in situ hybridization methods using biotin and digoxigenin (DIG) as labels for riboprobes. The methods were established using murine heavy chain constant region cDNA (MuCμ) antisense riboprobe on cytocentrifuge preparations of IgM- and IgG-synthesizing mouse hybridoma cells, normal rat BM cells and splenocytes. For detection of either label, enzymatic and fluorescent methods were compared.

The methods were developed by optimizing stringency washing conditions, background blocking, and for enzymatic detection, conditions for color development in the substrate.

Both types of detection methods and labels were successful in detecting μ mRNA in IgM synthesizing hybridoma cells and in normal cells with high copy number of the transcript such as $IgM^+$ plasma cells and plasmablasts.

The enzymatic detection of DIG label using anti-DIG antibody conjugated to alkaline phosphatase was also successful in the detection of low copy number transcripts in B lymphocytes and pre-B cells. The proportion of μ $mRNA^+$ cells in BM and spleen compared well with the frequency of μ $protein^+$ cells (about 30% and 40%, respectively).

In the past, several research groups have tried to detect μ mRNA in normal pre-B cells and B lymphocytes, but had to resort to radioactive techniques with probes with extremely high specific activity and exceptionally long exposure times, or manipulations such as LPS stimulation. To our knowledge we are the first who are successful in a rapid non-radioactive method for detection of μ mRNA in normal pre-B and B cells. We will apply the technique to study the frequency and location in BM of cells containing transcripts of molecules that may regulate B lymphocyte development.

**FZ 126** AUTOIMMUNE NZB MICE EXHIBIT LOSS OF BONE MARROW B2 (CONVENTIONAL) B CELL PRECURSORS WITH AGE, BUT RETAIN B1b B CELL PRECURSORS, Melinda S. Merchant, Beth A. Garvy, and Richard L. Riley, Dept. Microbiology & Immunology, University of Miami School of Medicine, Miami, FL 33101.

Autoimmune NZB mice lose bone marrow pre-B cells ($sIg^-$ $S7^+$ $B220^+$) and pro-B cells ($sIg^-$ $S7^+$ $B220^+$) in an age-dependent manner. We have analyzed the capacity of $B220^-$ T-cell depleted bone marrow cells from NZB mice to repopulate the splenic and peritoneal B cell compartments of sublethally irradiated (400R) C.B17 *scid* recipients. Three weeks post-transfer of B220 bone marrow cells from young (4-5 weeks old) NZB mice into C.B17 *scid* recipients, pre-B cells were reconstituted in recipient bone marrow in normal numbers. Recipient spleens contained significant numbers of B cells ($Ly1^-Mac1^-$), presumably conventional B2 B cells of NZB origin. In contrast, peritoneal cavity (PEC) B cells in these recipients consisted of both $Ly1^-$ $Mac1^-$ B cells and $Ly1^-$ $Mac1^+$ (B1b) B cells. C.B17 *scid* recipients of $B220^-$ bone marrow of older (24 weeks) NZB mice also possessed pre-B cells in their bone marrow, although pre-B cell numbers were significantly reduced when compared to recipients of young NZB $B220^-$ bone marrow cells. In contrast to the results obtained with NZB (young)-->C.B17 *scid* transfers, the spleens from NZB (old)-->C.B17 *scid* recipients generally possessed few B cells. However, PEC $Ly1^-$ $Mac1^+$ B cells recovered from NZB (old)-->C.B17 *scid* recipients were generally equivalent to those recovered from NZB (young)-->C.B17 *scid* recipients while PEC $Ly1^-$ $Mac1^-$ B cells were reduced. These results suggest that 1) NZB bone marrow $B220^-$ cells contain precursors for both B2 B cells, which populate spleen, and B1b B cells which populate the peritoneal cavity; 2) NZB mice lose bone marrow B lineage precursors cells with age; and 3) older NZB mice preferentially lose the capacity to repopulate splenic and PEC B2 B cells, but retain capacity to repopulate PEC B1b B cells. (Supported by NIH grant AI23350 to RLR).

**FZ 127** PROGRESSIVE, AGE-DEPENDENT INHIBITION OF PRE-B AND PRO-B CELL DEVELOPMENT IN AUTOIMMUNE NZB MICE AND SENESCENT BALB/C MICE. Richard L. Riley, Beth A. Garvy, Melinda S. Merchant, and Jeanne M. Elia, Dept. Microbiology & Immunology, University of Miami School of Medicine, Miami, FL 33101.

Decreased numbers of bone marrow small pre-B cells ($sIg^-$ $S7^-$ $B220^+$) are observed in young adult (2-10 months) NZB mice. In contrast, numbers of pro-B cells ($sIg^-$ $S7^+$ $B220^+$) do not decrease until approx. 14 months of age in these mice. The incidence of IL-7 responsive B lineage precursors (IL-7 CFU) decreases in parallel with the loss of $S7^+$ pro-B cells in NZB bone marrow. In normal BALB/c mice, reduction in small pre-B cells occurs much later in life (≥15 months), but again precedes loss of IL-7 responsive precursors. These results suggest that alterations in B lineage cell development are accelerated in the NZB as compared to the BALB/c strain, but in both instances B lineage abnormalities occur in 2 age-dependent phases: first, loss of pre-B cells occurs, followed later in age by loss of pro-B cells and IL-7 responsive B lineage precursors. In NZB mice, the initial decline in pre-B cells coincides with increased numbers of B1 ($Ly1^+$ and/or $Mac1^+$) B cells in the periphery, increased concentrations of serum Ig, and increased titers of Ig reactive with pre-B cell lines. As previously indicated (Kruger, M.G., Riley, R., Elia, J. 1992. Ann. N.Y. Acad. Sci. 651: 170), adoptive transfer of B cells from older NZB mice into (NZB x BALB/c)F1 recipients results in decreased numbers of small $sIg^-$ $B220^+$ pre-B cells in those recipients which show expansion of donor NZB B1 B cells. These findings suggest that the loss of pre-B cells in NZB mice may, in part, be a consequence of B1 B cell activation and expansion, possibly via production of pre-B cell reactive autoantibodies. (Supported by NIH AI23350 and American Federation for Aging Research grants to RLR).

FZ 128 B CELL LINEAGES INVOLVED IN THE IMMUNE RESPONSE TO PC-KLH. Alan M. Stall and Sandra M. Wells. Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, NY 10032

B-1 cells are able to respond to several T-independent antigens such as bacterial lipopolysaccharides and α1-3 dextran. It has recently been shown that B-1 cells can also mount an idiotypically-restricted T cell-dependent immune response to phosphorylcholine-keyhole limpet hemocyanin (PC-KLH). It is still unclear, however, to what extent, if at all, conventional B cells contribute to this response. Using combinations of classical and newly described cell surface markers we can now distinguish splenic B-1 and conventional B cells. In particular, the antigens CD23 (FceR) and BLA-1, define three distinct populations of splenic B cells. In contrast to most conventional B cells, cells of the B-1 lineage lack CD23 but express the BLA-1 antigen. Using these and other markers in BALB/c mice, we have identified two phenotypically and functionally distinct populations of B cells expressing antibodies detected by anti-VT15 MAb. This monoclonal Ab identifies all B cells expressing the $V_H1$ gene of the S107 family. One population consists of conventional B cells expressing both surface CD23 and IgD but lacking detectable BLA-1. In contrast, the second population displays the B-1 cell phenotype described above. In addition, these cells express the T15 idiotype characteristic of the BALB/c anti-PC response and contain all the PC reactive cells in the animal. Adoptive transfer studies confirm that this second population is derived from B-1 cells. These findings are discussed with respect to the activation of B-1 cells and competing theories of the generation of B-1 cells.

FZ 129 CHARACTERIZATION OF THE LIP-6 ANTIGEN, WHICH IS EXPRESSED BY MURINE BONE MARROW $B220^-$ B CELL PROGENTORS AND BY SUBPOPULATIONS OF MATURE PERIPHERAL B CELLS, Jeffery K. Taubenberger‡, Larry M. Lantz§, Kevin L. Holmes§, Laboratory of Pathology, NCI‡, Laboratory of Immunopathology, NIAID§, National Institutes of Health, Bethesda, MD 20892

The novel antigen, LIP-6, is expressed by $B220^+$ bone marrow pre-B cells, mature B cells and some myeloid lineage cells, but not by T cells or thymocytes. It is also expressed by 6-12% of $B220^-$ bone marrow cells. LIP-$6^+$ $B220^-$ Ly-$5^+$ bone marrow cells differentiate into $B220^+$ pre-B cells when sorted and cultured on S17 stromal cells (with IL-7) under Whitlock-Witte conditions. However, LIP-$6^+$ $B220^-$ Ly-$5^+$ bone marrow cells do not give rise to *in vitro* CFU-GM or BFU-e, suggesting a restriction to B lineage differentiation.

Mature B cells express variable, strain dependent, levels of LIP-6; the majority of splenic and lymph node B cells express low or intermediate levels of LIP-6. Three color analysis of Peyer's patches shows that $B220^+$ B cells are either LIP-$6^{bright+}$ MEL-14(L-selectin)$^-$ (14-19%), LIP-$6^-$ MEL-$14^+$(42-55%), or LIP-$6^-$ MEL-$14^-$ (22-40%). The LIP-$6^{bright+}$ MEL-$14^-$ population is unique to Peyer's patch.

Antibodies to LIP-6 induce the *in vitro* proliferation of spleen cells in both normal and *nu/nu* mice in a dose dependent manner. After 72 hrs, 75% of LIP-6 stimulated spleen cells were $B220^+$, compared with 95% in LPS cultures. Preliminary experiments suggests that this LIP-6 induced proliferation is not associated with an increase in intracellular $[Ca^{++}]$.

FZ 130 CD23+ AND CD23- MURINE B CELLS DIFFERENTIALLY RESPOND TO ACTIVATION STIMULI. Thomas J. Waldschmidt, Teresa M. Foy, and Randolph J. Noelle. Department of Pathology, University of Iowa College of Medicine, Iowa City, IA 52242 and Department of Microbiology, Dartmouth Medical School, Hanover, NH 03755

Our laboratory has previously established that CD23 (FcεRII) is an effective marker in distinguishing among a number of murine B cell populations. In the spleen, CD23 is expressed on follicular B cells and is absent on marginal zone B cells. In the peritoneum, CD23 is present on conventional B cells and is not expressed on Ly1/sister (B1) cells. With the use of the CD23 marker and fluorescence activated cell sorting, we are purifying splenic and peritoneal CD23 positive and negative B cell populations and testing for their response to a number of polyclonal stimuli. The results indicate that all B cell populations respond to LPS and T cell dependent interaction (using plasma membranes from activated Th cells), although to differing degrees. Unexpectedly however, only CD23+ B cells are capable of responding to soluble mitogenic anti-IgM antibody. CD23- B cells from both the spleen and peritoneum fail to enter cell cycle, and may respond in a negative fashion. These results suggest that different B cell subsets may be intended to respond to distinct stimuli, and therefore distinct classes of antigens.

FZ 131 IMMUNIZATION OF NEONATAL OR ADULT MICE WITH POLYVINYL PYRROLIDONE (PVP) ELICITS ANTIBODY PRODUCTION BY NEWLY GENERATED CD5+ (B1a) B CELLS. Alan C. Whitmore, Geoffrey Haughton and Larry W. Arnold. Department of Microbiology and Immunology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

PVP is a thymus-independent (TI2) antigen which is highly immunogenic in neonatal and adult mice. Athymic, nude mice respond vigorously but *Xid* mice not at all. After a single adult immunization, PVP-specific antibodies of IgM, all IgG and IgA isotypes can be found in the serum. PVP-specific antibody-producing cells can be detected in the spleen and peritoneum by the ELISAspot technique. We have produced and sequenced the immunoglobulin heavy and light chain V-genes from 16 PVP-specific hybridomas from a mouse immunized as an adult with PVP. Three of the hybridomas are of the IgG3 isotype, the rest are IgM. Four different $V_H$ genes have been found more than once (not always with the same $V_L$ gene) and 4 $V_H$ genes have been observed once. Six of the 16 hybridomas use the lambda light chain. In those cases where the germline V gene sequence is known or can be inferred from the complete identity of a group of clonally unrelated hybridomas, no somatic mutation has been observed. N region insertions are common. We have observed clonally related IgM and IgG3 PVP-specific hybridomas with identical $V_H$ regions, both in the germline configuration, suggesting that isotype switching and somatic hypermutation are not invariably linked.

Cell-sorting experiments indicate that the majority of B cells secreting anti-PVP IgM after adult immunization are $IgM^{hi}$, $b220^{lo}$, $CD5^+$. We have also constructed irradiation chimeras using allotype-marked donors of adult bone marrow and adult peritoneal cells. After immunization with PVP, all of the PVP-specific antibody is of the bone marrow donor allotype and more than 95% of the VH11 and VH12 idiotype-bearing antibody is of the peritoneal cell donor allotype. [In these chimeras, the bulk of the CD5-bearing B cells in the peritoneum are derived from the bone marrow donor.] We interpret these results in the light of two competing theories about the origin of B1 B cells: one which suggests that the existence of B cell subsets reflects the existence of distinct developmental lineages of B cells and the other that suggests that B lymphocyte heterogeneity is caused by differences in the quality and context of antigen encounter.

FZ 132 REDUCTION OF MURINE NATURAL KILLER CELL ACTIVITY IN VIVO PARTIALLY INHIBITS A SPECIFIC ANTI-TNP RESPONSE, J.A. Wilder, and D. Yuan, Department of Pathology, University of Texas Southwestern Medical Center, Dallas, TX 75235.

Our laboratory has previously reported that murine natural killer (NK) cells, and supernatents taken from NK cells grown in IL-2 for 6 days, can stimulate B cell proliferation and differentiation in vitro. Recently, we have begun to investigate the role of NK cells during the initial phases of a T-independent specific antigen response in vivo. Recipient $B_6D_2F_1$ mice were irradiated and depleted of NK cells by an i.p. injection of anti-NK 1.1 antibody. Subsequently, these mice received syngeneic B lymphocytes, immediately followed by immunization with TNP-LPS. The level of NK cell activity remaining after this treatment was monitored by a $^{51}Cr$-release assay performed on splenocytes from identically treated mice. Serum immunoglobulin levels were measured at various times after antigen injection by isotype specific ELISA or RIA. Anti-NK 1.1 treatment inhibited the specific response of these mice to TNP by an average of 35% as measured by serum IgM levels 6 days after antigen injection. These data suggest that NK cells may play a role in vivo, as well as in vitro, in amplifying B cell responses. We are currently investigating the kinetics of this inhibition by depleting NK cells at various times before and after antigen injection and B cell reconstitution.

FZ 133 ESTABLISHMENT OF IMMUNOGLOBULIN GENE TRANSFECTED B CELL LINES, Hiroshi YAMAMOTO and Yasushi MATSUURA, Division of Immunology, National Institute of Neuroscience, NCNP, Kodaira, Tokyo 187, Japan.

MOPC104E myeloma idiotype (M104E CRI) is immunogenic in inducing idiotype specific T cells. In order to investigate the idiotype-dependent T cell repertoire selection mechanisms, M104E heavy chain gene transfected B cell lines were established. M104E genomic-rearranged EcoRI fragment was cloned and sequenced. The deduced amino acid sequence was matched to the previous data. The genomic rearranged gene of the anti-idiotypic B cell line-derived hybridoma, HB19, was also cloned and sequenced. The sequence similarity of these two J558 family genes was highest among the family so far screened. Each EcoRI fragment of these was ligated with Ig-μ constant region gene and cloned into pSV2neo vector (pSVM104.M and pSVHB19.M). The constructs were transfected into A20/2J B lymphoma cell line (BALB/c origin) followed by G418 selection, and transfected cell lines, AME as pSVM104.M- and AHB as pSVHB.M-transfectants, were established. The transfectants expressed both μ-chains in the cytoplasm (by histochemical staining) and full-length μ-chain gene transcripts (by northern blotting). Several T cell lines (BALB/c origin) reactive to M104E protein were also established. Some of them exhibited significant proliferative response against both AME and AHB-series of transfectants but lower response against the control cell lines (pSV2neo transfected A20/2J cells). The results suggest that the T cell lines are recognizing transfected μ-gene product(s). These transfected cell lines are useful for the study of self Ig specific T cell repertoire selection mechanisms.

*Adhesion & Accessory Molecules*

FZ 134 HLA-DR IS INVOLVED IN THE INDUCTION OF DEHESION IN B CELLS. Els J.M. Ahsmann, Andries C. Bloem. Department of Immunology, University Hospital, Utrecht, the Netherlands.

A mAb (F5.17) was selected which interferes in the binding of human B lineage cells to adherent growing cells (endothelial cells, human and mouse fibroblasts). Specificity of mAb F5.17 for HLA-DR was shown by sequential immunoprecipitation studies and binding to HLA-DR transfectants. Since HLA-DR itself was not physically involved in the adhesion process, it was concluded that HLA-DR participates in transmembrane signaling, resulting in a reduced binding ability (de-hesion). The rapid de-hesion effect was mediated only through HLA-DR. MAbs directed against CD19 or the antigen receptor on B cells did not induce the inhibitory effect.

Induction of de-hesion with F5.17 or its $F(ab')_2$ fragments did not require $Ca^{2+}/Mg^{2+}$ nor metabolic energy. Treatment of the B cells by prior incubation with cytochalasin B or PKC inhibitors did not abrogate the HLA-DR transduced de-hesion. Incubation of the adherent cells with neuraminidase however, completely annihilated de-hesion. This indicates the involvement of a carbohydrate ligand on the adherent cells and suggests a LEC-CAM like receptor on the B-cells. Till now we have not been succesful in identifying this molecule.

HLA-DR can transduce signals resulting in homotypic aggregation of B cells[1]. Here we provide evidence that the same molecule is involved in the induction of de-hesion. This suggests that HLA-DR plays an important role in the regulation of adhesion of B cells.

1. Kansas GS, Tedder TF: Transmembrane signals generated through MHC Class II, CD19, CD20, CD39 and CD40 antigens induce LFA-1-dependent and independent adhesion in human B cells through a tyrosine kinase-dependent pathway. J Immunol 147:4094, 1991.

FZ 135 REGULATION OF INTEGRIN α5β1 EXPRESSION DURING B CELL DIFFERENTIATION
Thomas M. Birkenmeier, Washington University School of Medicine, St. Louis, MO 63110

Integrins are heterodimeric receptors that mediate cell-cell and cell-extracellular matrix interactions important for cell migration and differentiation. α5β1 is a receptor for the extracellular matrix protein fibronectin (FN). Adhesion of maturing lymphocytes to FN is important for the maturation process; however, these cells subsequently detach from fibronectin and move into circulation as they mature. Expression of α5β1 is controlled during B cell development—it is present on immature B cells, but absent from mature cells—suggesting that it controls the interaction of maturing lymphocytes with FN. I have examined the molecular mechanism controlling expression of α5 (subunit specific for α5β1) during B cell differentiation using model cell lines. The α5 gene promoter is hypomethylated in immature B cell and hypermethylated in mature cells, suggesting that methylation may control its activity. In support of this possibility, treatment of the mature B cell line Raji with 5-azacytidine activated α5 expression. Using in vitro methylation and transfection assays, two methylation-sensitive CpG dinucleotides that are critical for α5 promoter activity have been identified. One of these sites forms a unique protein-DNA complex. This site is methylated in mature B cells, but it is unmethylated in immature cells, suggesting that the methylation-specific protein that binds this site inhibits α5 gene transcription. I propose that this methylation-specific binding protein controls α5 gene expression during B cell differentiation.

In addition the α5 gene contains the consensus sequence for the Lyf-1 transcription factor, previously described in the TdT gene. I have demonstrated that this site acts as an enhancer for the α5 gene in fibroblasts. Interestingly, this activity does not require the INR sequence found in the α5 gene suggesting: (1) that transcription is initiating at a site distinct from the INR and (2) the INR may not be required for expression of α5 in all cell types.

**FZ 136** ICAM-1 IS INVOLVED IN TRANSMEMBRANE SIGNALING.

Andries Bloem, Ahsley Duits, Ger Rijkers*, Jan van de Winkel, Stoffer Looman, Martin van Horssen, Saskia Boom. Departments of Immunology of the University Hospital and the Wilhelmina Hospital for Sick Children*, Utrecht, the Netherlands.

Intercellular adhesion molecule 1 (ICAM-1, CD54) is a member of the Ig supergene family and participates in adhesive interactions between various hemopoietic and non-hematopoietic cells. In two different modelsystems a possible role for ICAM-1 in transmembrane signaling was investigated, employing different mAb directed against epitopes involved in cellular adhesion and located on the $NH_2$-terminal domain (domain 1) of ICAM-1.

It was found that antigen receptor transduced $[Ca^{2+}]_i$ mobilization in a Burkitt lymphoma B cell line could be inhibited upon co-crosslinking of ICAM-1. Furthermore the IL-6 production by LPS or IL-1β stimulated normal blood monocytes was effectively inhibited after crosslinking of ICAM-1. In both systems a possible role for Fc-receptors in the signaling process was excluded.

These data provide further support for the idea that molecules involved in adhesion also participate in signal transduction processes. In contrast to reported signaling through adhesion molecules, signaling through ICAM-1 results in the *down-regulation* of the generation of a second messenger ($[Ca^{2+}]_i$) and inhibition of IL-6 secretion. Present studies are directed towards the elucidation of the pathways involved in ICAM-1 signaling.

**FZ 137** EFFECT OF CONTACT MEDIATED SIGNALS ON REVERSAL OF ANERGY IN THE BKS-2 B CELL LYMPHOMA

Bondada Subbarao and Subramanian Muthukkumar, Department of Microbiology and Immunology and the Sanders Brown Center on Aging, University of Kentucky, Lexington, KY40536

Our previous studies showed that signals provided through the IgM receptor or elevation of calcium with ionomycin will induce anergy in the BKS-2 B cell lymphoma cells. Here we investigated the ability of T cell derived signals to rescue the BKS-2 cells from such anergy. Consistent with the two signal model of lymphocyte activation, culture of anergic cells with activated T helper cell clones reversed the growth inhibition of BKS-2 cells induced by anti-IgM or ionomycin. Although interlukin-5 was partially effective in such a rescue, activated T cells were more effective in completely overcoming the growth inhibition. Further, using transwell cultures we showed that cell contact mediated signals were important in the T helper cell induced reversal of growth inhibition induced by anti-IgM or ionomycin. Culture of BKS-2 cells with activated T cell clones for short term led to an increase in intracellular calcium levels in the BKS-2 cells. Currently, we are investigating the role of cell surface molecules such as CD40 and Lyb2 in this cell contact mediated signal transduction in BKS-2 cells.

(Supported in part by NIH grants AI 21490, KO4AG00422 and AG 05731 to B.S)

**FZ 138 THE HB4 MONOCLONAL AB RECOGNIZES A SUGAR EPITOPE BELONGING TO THE FAMILY OF α2,6-SIALYLTRANSFERASE GENERATED LYMPHOCYTE SURFACE ANTIGENS.**

De Lau, W.B.M.*, J. Kuipers*, H. Voshol#, J.C. Clevers*, and E.J.E.G. Bast*.

* Department of Immunology, University Hospital Utrecht, The Netherlands.

# Bijvoet center for biomolecular research, Utrecht University, The Netherlands..

Cells can regulate the specificity of the carbohydrate chains on their membrane-bound glycoconjugates by differential expression of glycosyltransferases. In lymphocytes the α2,6-sialyltransferase (α2,6-ST) has been demonstrated to be involved in expression of the epitopes recognized by the HB6, CDw75 and CD76 mAbs. Using expression cloning, we now could demonstrate that the HB4 antigen as found on a subpopulation of B - and NK cells, is another member of this family. Transient expression of α2,6-ST in COS cells generated a minor subpopulation of $HB4^+$ cells completely contained within the large $HB6^+$ population in line with a comparable overlap in expression on blood cells. Porcine thyroglobulin, an α2,6-$NeuAc^+$ glycoprotein was recognized by both mAbs in a neuraminidase sensitive fashion. This again underlined the close relationship of the HB4 and HB6 epitopes. It further implied that the mAbs reacted with oligosaccharide chains irrespective of the nature of carrier molecule. Using various proteinases and dMM inhibition of carbohydrate processing, we demonstrated that both epitopes were components of N-glycosylated membrane proteins, and that only a partial overlap occurred between their protein carriers on B cells. Sialic acid, a charged sugar residue found on terminal and side positions of complex carbohydrate chains, plays a pivotal role in the epitopes recognized by HB4, HB6, CDw75, and CD76. This makes a role of these distinct epitopes in biological recognition plausible.

**FZ 139** PRODUCTION OF SOLUBLE MURINE CD38 FOR STUDYING ITS BIOLOGICAL FUNCTIONS

J. Christopher Grimaldi, Satish Menon, Tony Muchamuel, Leopoldo Santos, Maureen Howard, DNAX Research Institute, Palo Alto, CA, USA

A 42Kd murine B cell antigen has been previously cloned. This antigen showed a 68% homology, at the nucleotide level, with human CD38. In addition this antigen has been shown to be important in the activation and proliferation of B cells. In order to further study the function of this molecule a soluble form was created by removing the transmembrane domain, located at the amino terminus, and replacing it with the CD8 leader followed by an 8 amino acid "flag" sequence. The soluble murine CD38 molecule has been expressed in L-cells. and purified to homogeneity by affinity chromotography using the commercially available M1 antiflag column. The soluble molecule is recognized by elisa and FACS using the antiCD38 antibody NIM-R5 and is recognized by western blot using M1 antiflag antibody. In additon, the soluble murine CD38 has been used to block the binding of NIM-R5 to full-length murine CD38 stable transfected L cells. Current efforts are focused on studying the function of CD38 using the soluble molecule in binding studies as well as several bioassay involving B cell activation.

**FZ 140** TWO FUNCTIONALLY DISTINCT EPITOPES ON MURINE CD40. Andrew Heath, Maureen Howard and John Gordon†, DNAX Research Institute, Palo Alto, CA 94304 and †The Medical School of Birmingham, UK.
We have produced a polyclonal antiserum and a monoclonal antibody to murine CD40. The anti-CD40 antiserum stimulates vigorous proliferation of normal B lymphocytes. In contrast, the mAb (11E11) failed to stimulate splenic B cells whether or not other signals were provided. When immobilized onto tissue culture plastic, 11E11 was found to be inhibitory for small B cell responses to either polyclonal anti-CD40 or anti-IgM; the response of large B cells to these stimuli was unaffected. Immobilized mAb to CD40 also left unchanged the capacity of polyclonal anti-CD40 to rescue CH31 B lymphoma cell from growth arrest induced by anti-IgM and, moreover, had no influence by itself on such inhibition: this was in spite of its ability to promote extensive clustering of CH31 lymphoma cells in response to polyclonal anti-CD40 or anti-IgM. These findings segregate the ability of CD40 to promote clustering from its role in B cell growth and survival. The differential, but interactive, behavior of the two antibodies employed in this study reveals the presence of two functionally distinct epitopes on murine CD40 and opens the possibility for the existence of two distinct ligands.

**FZ 141** CD44 EXPRESSION BY B CELLS: EVIDENCE FOR ALTERATIONS OF CD44 ISOFORM INDUCED BY B CELL ACTIVATION, Hiroyuki Hirano, Karen S.Hathcock and Richard J.Hodes, The Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892
CD44 is a glycoprotein expressed by various cell types including lymphoid cells. Recently, it has been demonstrated that some tumor lines reveal heterogeneity in CD44 resulting from differential glycosylation and alternative splicing of mRNA. Although lymphocytes are known to increase the amount of CD44 expressed on cell surface as a result of activation, it is still not fully analyzed whether normal lymphocytes express different isoforms of CD44. To analyze this possibility, we focused on B cells and examined the CD44 expressed by resting B cells or by B cells activated with LPS or with the Th cytokine IL-5. Both LPS and IL-5 induced quantitative increases of overall CD44 expression on the cell surface in comparison to the unstimulated splenic B cells and this was correlated with increase of mRNA level of CD44. B cell stimulation also induced changes in the apparent molecular weight of CD44 as determined by immunoprecipitation and gel analysis.
In order to determine whether activation of B lymphocytes induces differential CD44 expression at the level of alternative splicing of mRNA, RT-PCR analysis was conducted. The predominant CD44 mRNA species detected by PCR analysis was the same in all B cells examined. However, surprisingly, several species of larger amplified products were clearly observed from the cDNA of activated B cells. Furthermore, the induction of larger amplified products was also seen in activated cells from a B lymphoma line. The specificity of the observed PCR products was confirmed by probing with mouse CD44 cDNA and by restriction enzyme digestion. Thus, activation-dependent alterations in CD44 mRNA splicing are induced both in heterogenous splenic B cells and in homogenous B lymphoma cells.

**FZ 142** CD40 AND IL-4 IN THE REGULATION OF GERMLINE $\gamma 4$ TRANSCRIPTION, Michele D. Jumper, Judy B. Splawski, Peter E. Lipsky and Katheryn Meek, Department of Internal Medicine, UT Southwestern Medical Center, Dallas, TX 75235.
When human blood B cells are stimulated with antibodies to the CD40 molecule in the presence of IL-4, the relative amounts of IgG4 and IgE (%total Ig secreted) are markedly increased. To delineate the molecular events leading to immunoglobulin secretion in the CD40 system, we designed a PCR strategy to amplify both germline transcripts and mature transcripts. The PCR data showed that not only was there a bias toward secretion of IgG4 and IgE, there was also a noticeable induction of germline $\gamma 4$ and $\epsilon$ transcripts prior to the detection of mature transcripts. The co-regulation of IgG4 and IgE is not surprising for a number of reasons. First, they have functional homology -- monoclonal antibodies to either IgG4 or IgE induce histamine release from human basophils in vitro. In addition, the cryptic promoter sites located 5′ of the $\gamma 4$ and $\epsilon$ switch regions contain an evolutionarily conserved sequence (ECS) that is shared between mouse and man. Results from another group show that this region is important in the regulation of the germline $\epsilon$ transcript. To study the regulation of the germline $\gamma 4$ transcript, we have cloned the ECS into a CAT reporter construct to assay for promoter activity which is inducible by anti-CD40 and IL-4 stimulation. In combination with the transfection experiments, we plan gel mobility shift assays to look for specific proteins which might be induced by anti-CD40 and IL-4 stimulation. The combination of these experiments should help delineate the regulation of $\gamma 4$ germline transcription.

**FZ 143** MOLECULES INVOLVED IN THE HOMING OF B CELLS TO THE CHICKEN BURSA OF FABRICIUS, Emma Masteller, Kelvin Lee, Louise Carlson, Bronislawa Petryniak, and Craig B. Thompson. Howard Hughes Medical Institute and Departments of Internal Medicine and Microbiology/Immunology, University of Michigan Medical Center, Ann Arbor, MI 48109
During avian B cell development, immunoglobulin rearrangement is completed within the embryonic spleen by coincidental rearrangements of VH to DH heavy chain rearrangements and VL to JL light chain rearrangements. This process begins on embryonic day 10 and is completed by embryonic day 15. It occurs even in animals bursectomized at 2 1/2 days of development. However, since the avian immunoglobulin genes are encoded only by single V and J elements in both the heavy and light chain gene, this is not sufficient to generate an immunologic repertoire. For an immunologic repertoire to be generated, developing B cells must migrate from the spleen to the bursa of Fabricius where they are induced to proliferate and undergo gene conversion of their rearranged V gene segments. This proliferative phase of B cell development is dependent on the surface expression of the germline encoded immunoglobulin molecule. This suggests that there may be specific homing mechanisms involved in the migration of B cells from the spleen in which they arise to the bursa of Fabricius. Consistent with the involvement of a specific homing molecule in the traffic of splenic B cells to the bursa of Fabricius, we have found that bursal stem cells which seed the bursa of Fabricius specifically adhere to the bursal endothelium, and that this property is absent in mature bursal lymphocytes. Monoclonal antibodies have been identified which recognize these two distinct populations of cells, one of which blocks the ability of bursal stem cells to adhere to bursal stromal tissue. We are in the process of identifying this molecule and characterizing it as a potential homing receptor.

FZ 144 STIMULATION OF B CELLS WITH ANTIBODIES TO TWO DISTINCT EPITOPES ON THE HUMAN CD40 RECEPTOR. Staffan Paulie, Pia Björck and Sten Braesch-Andersen#, Department of Immunology, Stockholm University, 106 91 Stockholm, Sweden and #) Department of Pathology, Harvard Medical School, Boston, MA 02129

Antibodies to the B cell surface molecule CD40 are agonistic and have been shown to induce both proliferation and differentiation of human B cells. In the present study we have used a newly derived IgM Mab (17:40) to furher demonstrate the utility of antibodies in studies of CD40 function. As seen from binding experiments this Mab was directed to an epitope which is distinct from that recognized by most other CD40 antibodies (e.g. G28-5, S2C6 and mab 89). However, similar to these antibodies the 17:40 Mab mediated a variety of stimulatory effects on B cells including the induction of cellular adhesion and the costimulation of proliferation with TPA or IL-4. Furthermore, it cooperated with other CD40 antibodies to induce more vigorous responses than what was seen when single antibodies were used. These results show that antibodies do not have to interact at a specific site on the CD40 molecule and support previous observations that receptor crosslinking is an essential step in signal transduction.

By using a combination of the S2C6 and 17:40 antibodies we also developed a sensitive sandwich ELISA. This was used for the quantitation of CD40 in cell lysates as well as for the demonstration of shedded antigen in supernatants of CD40 expressing cells. Whether this soluble form of CD40 also displays biological activity and carries a regulatory function is presently being investigated.

FZ 145 ACTIVATION OF CD20 AND CD40 INDUCES A PROLIFERATIVE RESPONSE IN B CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA (BCP-ALL) CELLS.

Frans Smiers, Marleen van Paassen, Karel Hählen, Bob Löwenberg, Ivo Touw. Dr.Daniel den Hoed Cancer Center and Sophia Children's Hospital, Rotterdam, The Netherlands.

Regulation of proliferation of normal and leukemic BCP is a poorly understood process. The cytokines IL3 and IL7 may induce mitogenic responses in BCP-ALL cells, although sometimes only in combination with bone marrow stroma. The mitogenic responses of BCP-ALL cells are highly heterogeneous and in a significant proportion of cases no response to (combinations of) these stimuli is seen at all. Clearly, additional stimuli must be involved in the growth control of normal and leukemic BCP. In this study we tested in 15 cases of CD20 positive BCP-ALL whether leukemic BCP could respond to activation of CD20 by the antibody 1F5. In 4 out of 15 cases a significant increase of 3H-Thymidine incorporation was seen in response to stimulation with 1F5.

Six of these 15 cases were additionally tested for their expression of CD40 and their response to aCD40 antibody. All six cases showed membrane expression of CD40. A weak mitogenic response in four cases was seen only on day 3, but not on day 7. In two cases however the response to aCD40 was strong and increased on day 7. In two other cases 1F5 clearly synergized with aCD40. No synergistic effects of 1F5 or aCD40 were seen with IL3, IL7 and IL4. We further investigated whether responses to 1F5 and aCD40 depended on the maturation stage of BCP-ALL cells. No correlation could be found between immunoglobulin heavy- and light chain genes rearrangements, immunophenotype and responses to 1F5 and aCD40.

In conclusion we show that activation of CD20 and CD40 can evoke a mitogenic response in a number of BCP-ALL cases. However in other CD20 and CD40 expressing BCP-ALL cases activation of CD20 or CD40 has little or no effect even in combination with other growth factors. Whether this lack of responsiveness is due to a deficient CD20/CD40 signalling mechanism is currently under investigation.

FZ 146 T CELL-DERIVED COSTIMULATORY SIGNAL THROUGH THE CD40 MOLECULE RESCUES B CELLS FROM SURFACE IMMUNOGLOBULIN-MEDIATED CELL DEATH, Takeshi Tsubata, Jing Wu and Tasuku Honjo, Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto 606, Japan

Crosslinking of surface immunobloguln (sIg) on both immature and mature B cells seems to be able to induce apoptotic cell death, which is most likely involved in elimination of self-reactive B cells. B cell tolerance has been shown to be more easily induced in the absence of T helper cells, suggesting that T helper cells can modulate sIg-mediated apoptosis. To test this possibility, we cultured B lymphoma cells WEHI231 with anti-IgM in the presence or absence of T helper lines. While sIg multimerization by anti-IgM induced apoptosis, some but not all T helper lines rescued WEHI231 cells from cell death. The ligand for the CD40 molecule, CD40L, is expressed in the T helper lines capable of rescuing WEHI231 cells but not in the other lines. To test whether CD40L is resposible for the rescuing activity for WEHI231 cells, we introduced an expression vector for human CD40 into WEHI231, and treated the transfectant WEHI-hCD40 with anti-IgM and/or anti-human CD40. Stimulation by anti-CD40 prevented cell death of anti-IgM-treated WEHI-hCD40. Interaction of CD40L on T cells with CD40 on B cells may thus generate a signal that blocks sIg-mediated apoptosis. Absence or presence of the T cell help through CD40 may determine whether B cells are eliminated or activated upon interaction with antigens.

FZ 147 TWO ADHESIVE PATHWAYS TRIGGERED THROUGH CD19 IN B CELL PRECURSORS HAVE DISTINCT CHARACTERISTICS, Wen-Kai Weng, Tucker W. LeBien and Martin L. Wolf, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455

CD19 may play a role in B cell development by regulating the interaction between B cell precursors (BCP) and the bone marrow microenvironment. Epitope specific signalling through CD19 can induce cellular aggregation in both normal and leukemic BCP. We have detected an EDTA (10 mM) sensitive and insensitive response following CD19 signalling, both of which result in formation of cell aggregates. In a Traditional Aggregation (TA) assay, NALM-6 formed EDTA insensitive aggregates after 18 h incubation with anti-CD19. In contrast, IL-7 treated 1E8 ($sIg^+$ leukemic B cell line) and normal BCP ($CD10^+/sIg^-$) demonstrate an EDTA sensitive response to anti-CD19 in the TA assay. We have designed a Spin Down Aggregation (SDA) assay to evaluate the immediate anti-CD19 response. In the SDA assay, cells were preincubated with antibody for different time periods and then centrifuged (2400rpm, 1min) together. After gentle resuspension, cells were plated into 96-well plates and aggregation evaluated. Immediately (2 min) following addition of anti-CD19, both normal and leukemic BCP formed aggregates in the SDA assay. This immediate SDA response was EDTA insensitive and cytoskeleton dependent in all normal and leukemic BCP examined. However, this SDA response decays in 1E8 and normal BCP after 30-60 min preincubation with anti-CD19, yet persists in NALM-6. After IL-7 treatment 1E8 demonstrated a prolonged EDTA insensitive SDA response up to 4 h. IL-7 treated 1E8 also began to form the EDTA sensitive aggregates in the SDA assay 4-6 h after preincubation with anti-CD19. Besides the immediate EDTA insensitive SDA response, the normal BCP formed EDTA sensitive aggregates after 2 h of anti-CD19 preincubation in the SDA assay. Both EDTA insensitive and sensitive aggregation showed similar signalling features. They were both triggered with intact 25C1 (anti-CD19) or 25C1 $F(ab')_2$, but not 25C1 Fab or B4 (a different anti-CD19), and required continuous signalling to maintain aggregation. Therefore, we have shown that at least two adhesive pathways can be triggered via CD19 in BCP. An immediate response detectable by SDA assay was EDTA insensitive, and a second IL-7 dependent response was EDTA sensitive (which implicates the possible involvement of an integrin-like molecule). Both responses could play an important role in normal or aberrant B cell development.

FZ 148 CD19 TRIGGERED CELL AGGREGATION IN NORMAL B CELL PRECURSORS IS MEDIATED BY AN RGD SPECIFIC BINDING EVENT, Martin L. Wolf,Wen-Kai Weng,James B. McCarthy and Tucker W. LeBien,Department of Laboratory Medicine and Pathology,University of Minnesota,Minneapolis,MN 55455

The role of CD19 in normal B cell precursor (BCP) development may be in regulating interactions of the developing B cell with either stromal cells or extracellular molecules within the marrow microenvironment. In support of this hypothesis we have demonstrated that anti-CD19 mAb can induce in BCP at least two distinct adhesive phenotypes (EDTA insensitive and sensitive) resulting in homotypic aggregation. The EDTA sensitive/IL-7 dependent cell aggregation of BCP in vitro occurs in a fetal bovine serum (FBS) dependent manner. In the absence of FBS, no aggregation was observed after 18 h of anti-CD19 stimulation. Aggregation was maximal in the presence of 10% FBS and barely detected in 0.5% FBS. The FBS requirement could be replaced by serum-free medium which had been conditioned by bone marrow derived stromal cells or a hepatoma cell line. This suggested that homotypic aggregation was dependent upon factors in the complex biologic mixtures of serum or conditioned medium. In an effort to mimic known attachment factors present in serum which would be recognized by a possible integrin in an EDTA sensitive fashion, we tested an arginyl-glycyl-aspartyl (RGD) peptide-ovalbumin construct to determine if it could reconstitute the aggregation response in serum free medium. The RGD-ovalbumin was effective in reconstituting cell aggregation at doses as low as 10 ug/ml and maximum responses were detected between 100 and 200 ug/ml. The RGD-ovalbumin construct by itself did not induce cell aggregation in the absence of anti-CD19 stimulation, and the monomeric unconjugated RGD peptides were also without effect in the presence or absence of anti-CD19 stimulation. Identification of a possible integrin involved in the anti-CD19 induced responses has not been successful to date. We have been unable to block the anti-CD19 aggregation response using blocking mAb directed against the B1 or B2 integrin subunits and have been unable to detect the presence of a B3 integrin subunit on BCP. We now propose that the EDTA sensitive aggregation response may result from activation by anti-CD19 of an RGD receptor when RGD is presented in a multivalent form. The RGD containing molecule present in soluble form during our homotypic aggregation assays, may exist bound to other cell types or extracellular matrix within the bone marrow microenvironment. Therefore, signalling through CD19 could regulate RGD dependent attachment states of BCP during development.

*Expression and Selection of the Antibody Repertoire*

FZ 200 ROLE OF HOMOLOGY-DIRECTED RECOMBINATION : PREDOMINANTLY PRODUCTIVE REARRANGEMENTS OF VH81X IN NEWBORNS BUT NOT IN ADULTS, Reginald U. Chukwuocha and Ann J. Feeney, The Scripps Research Institute, Department of immunology, La Jolla, CA 92037

Vh J558 genes are underrepresented in early ontogeny, while 7183 and Q52 Vh genes are overutilized. The molecular mechanisms underlying this developmental regulation of Vh gene expression are not fully understood. We have shown that, in the absence of N region nucleotides, V-D and D-J junctions predominantly occur at regions of short homology. Based on analysis of where the short sequence homologies are located, we proposed that this homology-directed recombination will make mainly in-frame rearrangements with Vh 7183 and Q52 genes but make mainly out-of-frame rearrangements with VhJ558 in the neonate. In the adult, N regions would preclude formation of the predominant junctional sequences, and thus only 1/3 of all sequences should be in-frame. We tested this hypothesis using the 81X gene, a member of Vh 7183 gene family, since it does not undergo clonal expansion following rearrangement in pre-B cells and therefore, is not likely to be skewed by antigen selection. We observed that 69% of all 81X immunoglobulin rearrangements in fetal liver pre-B cells were productive. As predicted, most of the junctions in these sequences occur at the region of nucleotide homology between the gene segments. In contrast, only one-third of adult pre-B sequences were productive. This homology-directed recombination may play a role in the overexpression of Vh 7183 and Q52 in early life.

FZ 201 RESTRICTED REPERTOIRE OF VH12 REARRANGEMENTS IN NEONATAL AND ADULT B CELLS, Stephen Clarke, Jian Ye, and Suzanne McCray, Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

B cells specific for the hapten PtC are the single largest hapten specific group of B cells known in the mouse. These B cells belong exclusively to the B1 population, and are ligand selected. They express either of two VH/Vκ gene combinations, VH11/Vκ9 and VH12/Vκ4, and are highly restricted in VHCDR3. VH12 CDR3 in these antibodies is 10 amino acids and is encoded by a variety of DH genes and the JH1 gene. There is an invariant Gly in the 4th position encoded by DH. We have examined VH12 rearrangements in different lymphoid tissues by a PCR method that is independent of antigen specificity. VH12 rearrangements to JH1 in 1 day liver are also restricted, although to a lesser degree than in adult peritoneum; The encoded VHCDR3 in nearly 60% of productive (P) rearrangements is 10 amino acids in length and there is a marked bias for the use of a subset of DSP2 genes. A Gly or Asn is often found in the 4th VHCDR3 position. This bias is less evident in 18 day fetal liver. These data suggest that selection for PtC binding cells probably begins before birth, and likely involves both the selective expansion of certain clones and the elimination of most cells with P VH12 rearrangements. Examination of VH12 rearrangements in adult tissues suggests the existence of few B cells with P VH12 rearrangements that cannot be attributable to selection for PtC binding. This includes an analysis of VH12 rearrangements to JH genes other than JH1, and of splenic B cell populations depleted of PtC binding cells. Thus, elimination of P rearrangements continues in the adult, and appears to eliminate P VH12 rearrangements in the B2 population.

FZ 202 SEQUENCES OF CLONED HEAVY AND LIGHT CHAIN GENES OF HUMAN RHEUMATOID FACTORS (RF) DERIVED FROM PERIPHERAL BLOOD OF PATIENTS WITH RHEUMATOID ARTHRITIS (RA) - CORRELATION WITH IDIOTYPIC AND ANTIGENIC SPECIFICITY. A. Davidson, K. Youngblood, S. Artandi, N. Agostino *S. Morrison and V. Bonagura. Albert Einstein Coll. of Med., Bronx N.Y. 10461. *UCLA., CA.90024

We report the complete amino acid sequences of 11 IgM RF derived by EBV transformation of peripheral blood lymphocytes from 2 RA patients. The RF light (L) chains were encoded by members of the VkI (2/11), VkII (1/11) and VkIII (8/11) gene families. The heavy (H) chains were encoded by members of the VH1 (5/11), VH3 (2/11) and VH4 (4/11) gene families. The H chain D regions were highly variable in length and sequence. Of the RF using VkIII genes, 4 were encoded by Humkv325, 2 by Humkv328 and 2 by V3g. 3 of 4 antibodies using Humkv325 were +ve for the Wa id. The fourth antibody had somatic mutations in CDR1 and was associated with a VH4 encoded heavy chain. Neither of the antibodies using Humkv328 was +ve for the 6B6.6 id. One of the V3g encoded antibodies was +ve for the 4C9 id.

Comparison of the 4 VkIII sequences with the corresponding germline genes cloned from a PCR amplified VkIII library from one patient revealed somatic mutations in the CDRs. Comparison of the 5 VH1 sequences with VH1 germline genes cloned from both patients showed that 3 were derived by somatic mutation from the 51P1 gene, 1 from the 1-1 gene and 1 from a newly characterized VH1 gene.

Antigenic specificity was determined using genetically engineered chimeric IgG antibodies. While the dominant epitope lies in the CH2-CH3 interface, some RFs displayed unique specificities. The Wa +ve antibodies recognized a common region on Fc, however for the other lines fine specificity did not correlate with VH or VL gene usage .

Peripheral blood RF are similar to synovium derived RF from RA patients in their heterogeneity of fine antigenic specificity, diverse gene usage and accumulation of somatic mutations. Differences in antigenic/idiotypic specificity between monoclonal and polyclonal RF may be accounted for by diverse VH/VL usage and combinations, by somatic mutations and/or by diversity in the H chain D regions.

FZ 203 **Transcriptional Regulation of the Murine mb-1 Gene,** Robert C. Fisher, Andrew L. Feldhaus, David Mbangkollo, Kara L. Arvin, Christopher A. Klug and Harinder Singh. Howard Hughes Medical Institute and the University of Chicago, Chicago, IL 60637

The mb-1 gene product is an important component of the pre-B and B cell antigen receptors. Activation of the mb-1 gene appears to represent one of the earliest regulatory events in B cell differentiation since the gene is expressed in pro-B cells that have not initiated heavy chain gene arrangement. To explore the gene regulatory hierarchy responsible for B cell commitment we are analyzing transcription factors that regulate expression of the murine mb-1 gene.

Mb-1 belongs to a group of B cell-specific genes including V preB, λ5 and B29 whose promoters lack a TATA box and initiate transcription from multiple sites. A promoter region was identified and shown to contain two regulatory domains. The distal domain was shown to be active in a cell type and stage-specific manner Further deletional analysis of the distal domain identified a 25 bp element that binds a cell-type and stage-specific factor (BLyF) that mirrors the expression pattern of the mb-1 gene ( Feldhaus et. al., MCB, 1992, p1126-1133). DNase I hypersensivity analysis was used to scan the mb-1 locus for additional regulatory elements since the isolated mb-1 promoter region is weakly active in the absence of a heterologous enhancer. A tissue-specific and a ubiquitously present hypersensitive site has been detected 3' of the mb-1 gene. The region containing both hypersensitive sites is currently being tested for its ability to promote mb-1 transcription in B lineage cells.

FZ 204 QUANTITATIVE STUDIES OF B CELL REPERTOIRES IN PRIMARY AND PERIPHERAL LYMPHOID TISSUES BY LIMITING DILUTION AND FLUORESCENT in situ HYBRIDIZATION, R.A. Goldsby, K.S. Ravichandran & B.A. Osborne, Dept. of Biology, Amherst College, Amherst, MA 01002.

Using LPS-mediated limiting dilution analysis and a panel of antigens (DNP, PC and mouse RBCs), we show that the repertoire of specificities for these antigens generated in bone marrow is distributed without bias to peripheral tissues (spleen & Peyer's patches). Comparison of animals of different ages revealed that there was a two-fold increase in the generation and distribution of B cells reactive with autologous mouse red blood cells in older mice compared to young ones. Using a novel technique that employs fluorescent *in situ* hybridization and flow cytometry, we have also compared the usage of three $V_H$ gene families (7183, J558 & S107) in large numbers of single B cells from these mice. In adult mice, no significant difference was observed in the expression of these families in bone marrow and peripheral tissues (spleen & Peyer's patches). However, a two-fold increase in the use of $V_H$ 7183 in neonates as compared with adults was observed. In the case of adult BALB/c mice, our data show that for the antigens and $V_H$ families examined, there is little, if any, difference in the repertoire of B cell specificities or $V_H$ family usage between bone marrow and the peripheral lymphoid organs examined. However, specific and selective changes do occur in both $V_H$ gene usage and antibody frequencies during murine ontogeny.

FZ 205 A SECOND NATURAL LIGAND FOR HIV gp120, Lee Goodglick, Linda Berberian, and Jonathan Braun Department of Pathology and Laboratory Medicine, UCLA School of Medicine, Los Angeles, CA 90024

AIDS is characterized by dysfunction and deletion of immune cells including T cells expressing CD4 and B cells expressing immunoglobulin (Ig) VH3 gene products. Deletion of CD4 T cells is postulated to result from the binding of HIV (via gp120) to CD4. Analogously, we postulated that deletion of B cells expressing VH3 Ig might also depend on direct binding of HIV to these cells. We therefore tested whether gp120 bound to a subpopulation of normal B cells. Human mononuclear tonsil cells from seronegative individuals were incubated with gp120 and analyzed by flow cytometry. Three to six percent of the B cells ($CD19^{bright}$) bound to gp120. The majority (72%) of the B cells which bound to gp120 expressed VH3 Ig. gp120 did not bind to these B cells via CD4 since a) tonsil B cells had no detectable CD4 surface protein, b) the anti-CD4 antibody Leu3a did not inhibit binding of gp120 to this B cell subpopulation, and c) nonglycosylated gp120, which does not bind CD4, bound to the B cells. We hypothesized that surface Ig bound gp120. To test this 94% of membrane-bound Ig was removed by pretreating cells with anti-Ig monoclonal antibody. Such treatment virtually abolished the binding of B cells to gp120. We directly investigated the binding of Ig to gp120 in a cell-free system by ELISA. There was a dose-dependent binding of normal, HIV-seronegative serum IgM to 50 ng of gp120. Significant binding was detected using as little as 5-10 ng of serum IgM. Binding of IgM to gp120 was ≥10 fold greater than IgG. To determine the contribution of VH3 Ig to this subset of IgM, we divided serum into IgM which bound and which did not bind *S. aureus* protein A. Protein A is an Ig superantigen which binds primarily to VH3. The fraction of IgM which bound to protein A contained the majority of the gp120-binding activity. In conclusion, a subset of membrane-bound and soluble VH3 Ig binds to gp120. We hypothesize that HIV may deplete VH3 B cells by a superantigen-like mechanism.

**FZ 206** STUDIES OF IMMUNOGLOBULIN LIGHT CHAINS IN THE NURSE SHARK, GINGLYMOSTOMA CIRRATUM,
Andrew Greenberg and Martin Flajnik, Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, Fl 33136.

While mammals possess both kappa(κ) and lambda(λ) light chain isotypes, the point in evolution in which both classes arose is still not clear. In order to address this question, we performed anchored PCR on nurse shark spleen cDNA using oligonucleotides which would preferably amplify C-1 set Immunoglobulin superfamily members. Using this approach, we were successful in isolating the shark equivalent of mammalian κ light chains. Since only light chains of the λ isotype had previously been isolated so far, this indicates that the divergence of two light chain classes from a common rearranging precursor preceded the emergence of cartilaginous fishes some 400-450 million years ago. Southern blot analyses with Vκ and Cκ probes yield multiple bands for each probe, and we have found by PCR that the Vκ segment is separated from the Jκ segment by only 550 bp. These results suggest a gene organization of V-J-C for the shark κ locus, similar to that described for the λ locus for sharks. Since both κ and λ loci have a similar gene organization in the nurse shark, this indicates that the primordial light chain may have had a gene organization of V-J-C. In separate experiments, we have isolated both λ isotypes which have been described in two other shark species. Southern blots analyses of these light chains reveals few bands, and both λ light chains appear to be linked on a 6.5 kb fragment. This differs from what has been described for the λ light chain loci in other sharks, in which there are numerous V and C segments; thus the λ locus appears to be dynamic in its evolution and has undergone species-specific contraction and expansion in its gene segments.

**FZ 207** DISTINCT PATTERNS OF $V_H$7183 GENE UTILIZATION AND D-REGION STRUCTURE DURING B CELL DIFFERENTIATION,
François Huetz, Leif Carlsson and Dan Holmberg, The Unit for Applied Cell and Molecular Biology, University of Umeå, Umeå, Sweden

We have recently reported that the establishment of B cell repertoires in ontogeny involves cellular selection of individual $V_H$7183 genes. We here analyze the repertoire of $V_H$7183 rearrangements isolated from different stages of B cell differentiation in adult mice. The nucleotide sequence analyses of $V_H$7183-D-$J_H$ rearrangements derived from large pre-B cells (B220$^+$, $\mu^-$), small pre-B cells (B220$^+$, $\mu^-$) and mature B cells (B220$^+$, $\mu^+$) isolated from adult bone marrow (BM) revealed a sequential accumulation, among functional rearrangements, of D-segments of the FL16 family and a depletion of D-segments using the second and the third reading frame (RF). One member ($V_H$7183.1) of the $V_H$7183 gene family was utilized in 60%-80% of the rearrangements of all populations analyzed. In neonates the majority of the rearrangements utilizing this gene was found to be functional. In contrast, more than 96% of the $V_H$7183 rearrangements isolated from adult spleen were non-functional. These data provide evidence for cellular selection of $V_H$-regions acting at different points of the B cell differentiation pathway and at the transition of B cells from the BM to the periphery.

**FZ 208** ANALYSIS OF THE EXPRESSED ANTIBODY REPERTOIRE BY SINGLE CELL PCR OF FACS-SORTED B CELLS,
Aaron B. Kantor, Cynthia E. Merrill*, Leonore A. Herzenberg & Jan L. Hillson*, Stanford Univ., Stanford, CA 94305, *Univ. of Washington, Seattle, WA 98195.

We distinguish three murine B cell lineages (PNAS, 1992, 89:3320 and refs. therein): conventional B cells (B-2 cells), pwhich develop late and are continually replenished throughout life from progenitors in adult bone marrow; B-1a cells (Ly-1 (or CD5) B cells), which develop early and maintain their numbers by self-replenishment; and B-1b cells (Ly-1 B "sister" cells), which share many of the properties of Ly-1 B cells, including self-replenishment and feedback regulation of development, but can also readily develop from progenitors in adult bone marrow. Several studies indicate that the expressed repertoire of mature B-1a and conventional B cells differ with respect to VH gene usage and the extent of N region insertions. There is no information on B-1b cells. Earlier methods of repertoire analysis, which include generation of hybridomas, in situ hybridization with VH probes and the amplification of cDNA from polyclonal cells, are not necessarily representative of the populations studied. Here, we report a method for making cDNA from individual, unstimulated, FACS-sorted murine and human B cells that permits the recovery and amplification of up to 90% of the cells and analysis of transcripts of multiple genes from a single cell. Cells are sorted at one cell per tube, lysed in hypotonic media containing RNAse inhibitor, and cDNA prepared with random hexanucleotide primers. The murine cDNA is amplified with primers homologous to the 5' portion of Cμ and to a conserved consensus region in framework 1 of VH. Amplification of cDNA from hybridomas representing 13 murine VH families and the high recovery rate demonstrates that the procedure is void of bias for particular rearrangements. B-1a, B-1b and conventional B cells from the Balb/c peritoneum are bulk sorted based on size, viability and the expression IgM, IgD, and Ly-1. Following reanalysis, single cells are sorted and snap frozen. We are analyzing the VH genes of these B cell subsets.

**FZ 209** A STRUCTURAL AND MOLECULAR MODEL FOR T15-IDIOTYPE DOMINANCE,
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Antibodies bearing the T15-idiotype (Id) dominate the murine immune response to phosphocholine (PC). Analysis of the relative antigen-binding affinity of antibodies derived from V1:DFL16.1:$J_H$1 ($V_H$1) germ-line and N-region-derived variant heavy- (H) chains and κ22, κ24, and κ8 light- (L) chains demonstrates that the T15H:κ22L (T15) antibody binds PC at least 20 to 40 times better than other antibodies derived from alternate germ-line forms of the $V_H$1 H-chain and κ22, κ24, or κ8 L-chains. To achieve avidities in the same range as the T15 antibody, κ24 and κ8 L-chain containing antibodies must have H-chains derived from variant N-region or somatically mutated $V_H$1 genes. Single amino acid differences at the VD-junction of the various germ-line and N-region variant $V_H$1 H-chains dictate which L-chain can associate with the H-chain to produce a PC-specific antibody. Thus, κ22 requires the Asp 95H, κ24 requires the insertion of Ala or another residue at the VD-junction, and κ8 functions optimally with the Asn 95H variant in formation of PC-binding antibodies. Several H:L combinations give rise to T15 and M167-Id$^+$ antibodies that lack specificity for PC. Based on these observations, we propose a molecular model involving both preferential gene rearrangement and antigen-driven B-cell selection to explain T15-id dominance in the immune response to PC. In the absence of N-region diversification, large numbers of neonatal B cells bearing the high affinity T15H;κ22L sIgM receptors would be selected and expanded by autologous or environmental PC-antigen into the long-lived peripheral B cell pool. This model has been tested using transgenic mice expressing $V_H$1 variant H-chain genes. The B cells selected and amplified in these mice are both id$^+$ and PC-specific, while id$^+$, non-PC-binding clones are not selectively amplified. These PC-specific B cells express the transgene-encoded H-chain in association with the appropriate endogenous L-chain required to generate a PC-specific antibody.

FZ 210 THE ANTIBODY BINDING SITE IS THE PRODUCT OF A NESTED GRADIENT OF DIVERSITY. Perry M. Kirkham, Rotem A. Elgavish, Leif Carlsson, Dan Holmberg, and Harry W. Schroeder, Jr. Departments of Medicine and Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294-3300.

Mammalian immunoglobulin VH families can be grouped into three distinct clans based upon sequence and structural conservation of the first framework (FR) interval. VH gene segments belonging to the same family share their own unique FR 3 structure. Within families, individual gene segments have been associated with distinct antigen specificities. In order to gain insight into the structural significance of conserved clan and family structure and individual gene segment variation, we performed molecular modelling and multi-sequence comparisons of known heavy chain variable domains. The FR 1 intervals that encode the three VH clans, and their L chain counterparts, form a conserved ring of residues that separates the constant from the variable domains. The family-specific intervals that include portions of FR 3 and CDR 2 are bounded on one side by the clan-specific intervals, and on the other confine the V-encoded CDR 1 and CDR 2 portions of the antigen binding site. Within a given family, individual V gene segments sequences primarily diverge in the sequence and structure of these CDR 1 and 2 regions. Due to the solvent exposed nature of these CDR loops, it is typically assumed that variant CDR amino acids alter antigen binding due to direct antigen contact. However, molecular modelling of the 25 known members of the mouse VH 7183 family revealed instead that half of the most variable amino acid residues interact directly with the CDR3 regions of both the H and L chains and are unlikely to come into direct contact with antigen. Thus the antigen binding site is the product of a nested gradient of diversity: the highly conserved clan-specific external ring surrounds a family-specific ring that confines the V encoded CDR 1 and 2 portion of the antigen binding site, that in turn surrounds the paired CDR 3 regions that are the unique product of an individual rearrangement event. Our results provide a structural context for theories that address preferential VH-VL pairing, and preferential use of VH gene families and individual VH gene segments in the immune response.

FZ 211 IMMUNOGLOBULIN HEAVY CHAIN GENE $V_H$-D JUNCTIONAL DIVERSITY AT DIAGNOSIS IN PATIENTS WITH ACUTE LYMPHOBLASTIC LEUKEMIA, *Geoffrey R. Kitchingman, Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, Memphis, TN 38101*

Acute lymphoblastic leukemia (ALL) represents the clonal outgrowth of transformed hematopoietic progenitor cells. During the course of experiments designed to analyze residual disease in patients undergoing chemotherapy, we PCR amplified, cloned and sequenced rearranged antigen receptor genes from DNA prepared from leukemic cells obtained at the time of diagnosis. We have found that blast cells in some cases of B precursor cell ALL contain immunoglobulin (Ig) heavy chain gene rearrangements with considerable diversity at the junctions of the variable ($V_H$), diversity (D) and joining ($J_H$) regions. This diversity consists of heterogeneous nucleotide sequences at the $V_H$-D, and less frequently, the D-$J_H$, junctions. In two cases, different $V_H$ segments were attached to the same D-$J_H$ rearrangement. At least three mechanisms may produce these changes in different cases: (1) continuing rearrangement of the heavy chain gene, in some cases by $V_H$ addition to a pre-existing D-$J_H$, (2) $V_H$ replacement, and (3) an open and shut mechanism. Regardless of the operative mechanism, it is clear that Ig heavy chain gene rearrangement can evolve during clonal outgrowth of the leukemic clone, suggesting the presence of an active VDJ recombinase in a high percentage of ALLs. An active recombinase in the rapidly growing leukemic cell population could lead to genomic instability.

In all cases studied, there was a much higher than expected frequency of nucleotide sequence changes in the $V_H$ segment, ranging from 0.22 to 2.4%. Control experiments ruled out the possibility of artifactual generation of these results, leading us to conclude that somatic mutation is occurring in early B cell ALLs. The nucleotide changes are distributed along the whole length of the $V_H$ gene segment, and do not necessarily cluster to the complementarity-determining regions. The possibility exists that a complex of the mu heavy chain and the pseudo-light chain proteins lambda5 and Vpre-B on the cell surface is responsible for transmitting the signal to begin hypermutation.

FZ 212 DELETION MAPPING OF Ig $V_H$ GENE SEGMENTS PREFERENTIALLY EXPRESSED IN EARLY HUMAN B CELL ONTOGENY, Ton Logtenberg, Saskia B. Ebeling, Karine E. Akkermans-Koolhaas and Mieke E.M. Schutte. Department of Immunology, University Hospital Utrecht, Utrecht, The Netherlands.

In humans, expression of a restricted set of Ig $V_H$ genes has been observed in 15 and 19 week old fetal liver. Due to the complex organization of the human $V_H$ locus these data have, on the most part, been difficult to interpret in the context of chromosomal position of $V_H$ segments. We have addressed this issue by generating a panel of heterohybridomas containing a single rearranged human $V_H$ locus. Southern blotting analysis of the $V_HDJ_H$ rearrangements in these cell lines yielded a deletion map that confirmed and further refined previously reported maps of the human $V_H$ locus. In addition, the deletional analysis permitted us to determine the chromosomal position of a number of developmentally-restricted $V_H$ gene segments. The results demonstrate that the $V_H$4.21, 1-9III and 58P2 gene segments map at respectively 500, 850 and between 500 and 800 kb 5' from the cluster of $J_H$ minigenes. We conclude that $J_H$-proximity is not a major determinant of the restricted $V_H$ gene utilization pattern in early human B cell ontogeny.

FZ 213 BIASED UTILIZATION OF $V_K$ EXONS AND PREFERENTIAL $V_K$-$J_K$ RECOMBINATIONS IN EARLY ONTOGENY, Carmen A. Medina and Judy M. Teale, Department of Microbiology, The University of Texas Health Science Center, 7703 Floyd Curl Dr., San Antonio, TX 78284-7758

To determine the extent of kappa chain diversity in the pre-immune repertoire early in development, κ cDNA libraries were analyzed from 15 day fetal omentum, 18 day fetal liver, and 3 week old bone marrow. An anchored PCR approach was used to avoid bias for particular $V_K$ families. From the sequence analysis of 28 bone marrow clones, 10 different families and 20 unique $V_K$ genes were identified. In contrast the $V_K$ expression in the fetus is highly restricted and clearly differs from the broader distribution seen in 3 wk bone marrow. Although several $V_K$ families were represented in the fetal library including $V_K$9, $V_K$10, $V_K$4,5, $V_K$8, and $V_K$1; 1 or 2 members of individual families were observed repeatedly. The fetal liver and fetal omentum libraries were found to be largely overlapping. Given the $V_K$ families/exons identified in the fetal sequences, κ rearrangements in the early repertoire appear to be predominantly by inversion. Importantly, the fetal repertoire was further restricted by dominant $V_K$-$J_K$ combinations such as $V_K$4,5-$J_K$5, $V_K$9-$J_K$4, and $V_K$10-$J_K$1. Since in some cases independent rearrangements could be established, the results indicate a bias for particular $V_K$-$J_K$ joins. The results also suggest that clonal expansion/selection in the fetal repertoire takes place after light chain rearrangement as opposed to at the pre-B cell level in the bone marrow. The restriction observed in κ light chain expression together with known restrictions in gene usage and junctional diversity at the heavy chain level indicate a remarkably conserved fetal repertoire.

**FZ 214** DEVELOPMENTAL REGULATION OF DH AND JH GENE SEGMENT REARRANGEMENT IN MAN. Frank Mortari, Cesar Nuñez, Fred E. Bertrand III, and Harry W. Schroeder Jr. Departments of Medicine and Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294-3300.

The production of low affinity, self-reactive antibodies appears to be a necessary property of the developing immune response, and fetal serum is enriched for these "natural" autoantibodies. Fetal H chain variable domains are characterized by preferential use of a small subset of highly conserved VH, DHQ52, and JH3 & 4 gene segments. These limitations reduce the diversity of the H chain CDR 3 region that helps form the center of the antigen binding site. High affinity, "pathologic" autoantibodies are enriched for the same set of VH gene segments, but their CDR 3 regions rarely contain DHQ52 and JH3. Intead, these Ig frequently contain DXP to JH 4, 5 & 6 rearrangements. The intermediate products of H chain rearrangement, DJ transcripts, cannot form antigen-selectable product, yet they provide a measure of the relative frequency of D and J gene segment rearrangement. In order to gain insight into the timing and the mechanisms that allow production of high affinity antibodies, we examined DHQ52- and DH DXP-containing transcripts from spleen, normal PBL, fetal liver mononuclear cells, and sorted pre-B and B cells from fetal, juvenile, and adult bone marrow. In fetal liver of 54 through 76 days gestation, DHQ52 transcripts were abundant and DH DXP transcripts were rare. This is in spite of the fact that there are at least five members of the DH DXP family and only one member of the DHQ52 family. In bone marrow at 19 weeks gestation, DHQ52 and DXP transcripts were of similar abundance. In contrast, DH DXP-containing transcripts were prominent and DHQ52-containing transcripts were rare in all "mature" samples tested, including adult bone marrow and the bone marrows of two patients with X-linked agammaglobulinemia. Sequence analysis revealed extensive N region addition and preferential utilization of JH 4, 5 & 6 in these post-natal transcripts. These findings support the hypothesis that developmental control of the antibody repertoire reflects, at least in part, a mechanism of ordered gene segment rearrangement. Furthermore, these findings suggest that transition towards a "mature" heavy chain repertoire begins late in the second trimester.

**FZ 215** B LYMPHOCYTE TOLERANCE THROUGH ANTIGEN RECEPTOR EDITING, David Nemazee, Susan Tiegs and David M. Russell, Division of Basic Research, Department of Pediatrics, National Jewish Center, Denver CO 80206.

We have previously generated immunoglobulin gene transgenic mice using DNA contructs encoding the IgM and IgD forms of the anti-MHC class I antibody 3-83, which reacts with H-2 $K^k$ and $K^b$, but not $K^d$ molecules. On an H-$2^d$ background these mice have nearly normal numbers of peripheral B-cells and demonstrate impressive heavy and light chain allelic exclusion with >95% of the B-cells expressing only transgene-encoded immunoglobulin. On an H-$2^k$ or H-$2^b$ background, in which autoantigen recognized by the 3-83 antibody is present, B-cells with the transgene-encoded, autoreactive specificity are deleted from the peripheral lymphoid organs and these mice have decreased numbers of peripheral B-cells relative to H-$2^d$ transgenic mice. Only immature B cells in the bone marrow bear the autoreactive specificity as detected with the 54.1 anti-clonotype antibody.

We have found that autoreactive B-cells encountering $K^k$ or $K^b$ antigen in the bone marrow (central deletion) are induced to express V(D)J recombinase. This results in specific rearrangement of light chains, but not of heavy chains. This induced rearrangement is specific to centrally-deleting mice because peripherally-deleting mice (3-83 mice bred with MT-$K^b$ transgenic mice that express $K^b$ only in the periphery) have no recombinase upregulation relative to non-deleting mice. In addition, the numbers of λ-light chain bearing cells in the centrally-deleting mice is elevated ~10-fold over non-deleting or peripherally-deleting mice. A number of controls were done to rule out feedback induction. We could clearly demonstrate that the cells in the bone marrows of the centrally-deleting mice that expressed recombinase were $sIgM^+$/$idiotype^+$ cells. Because secondary V-to-J and RS rearrangement in kappa light chains can efficiently inactivate, and sometimes replace, active $VJ_\kappa$'s by nested rearrangement, we have proposed that secondary light chain rearrangement in autoreactive bone marrow B-cells can alter the antigen receptor, rendering the cell non-autoreactive. This receptor editing model argues that in the primary lymphoid organs receptor selection, rather than clonal selection may occur.

**FZ 216** IMMUNOGLOBULIN $V_H$ USAGE ANALYSIS BY FLUORESCENT IN SITU HYBRIDIZATION AND FLOW CYTOMETRY, B. A. Osborne, K. S. Ravichandran, A. S. Semproni and R. A. Goldsby, Molecular and Cellular Biology Program, University of Massachusetts and Department of Biology, Amherst College, Amherst, MA 01003

We have devised a flow cytometry-based fluorescent *in situ* hybridization assay that permits analysis of gene expression in a large number of single cells. In this technique, fixed and permeabilized cells are incubated with biotinylated RNA probes and by means of a fluorescently labeled second-step reagent, the cells are analyzed by flow cytometry. Using this approach, we demonstrate that immunoglobulin $V_H$ gene expression may be analyzed among individual cells using $V_H$ family-specific probes. The technique has a high degree of accuracy in detecting the fraction of cells expressing a specific message in a population and is sensitive enough to detect immunoglobulin message in LPS activated B cells. This method has been used to monitor gene expression in homogenous and heterogenous cell populations. It also allows concurrent analysis of cell surface proteins and gene expression through the use of two-color flow cytometry. Data demonstrating sensitivity and specificity of this technique will be presented.

**FZ 217** κ LIGHT CHAIN REARRANGEMENT IN FETAL LIVER DEVELOPMENT, D. A. Ramsden, C. J. Paige, and G. E. Wu, Dept. of Immunology. University of Toronto, Canada, M5S 1A8.

The predominant site of B cell development in the embryonic mouse is the fetal liver. Enumeration of rearrangements from fetal liver on successive days of gestation allowed us to address questions regarding the relationship between immunoglobulin rearrangement and the other events in B cell development. We developed a sensitive (detects <5 rearrangements /$10^5$ genomes), semi-quantitative PCR assay to enumerate κ light chain rearrangements from fetal liver cells, from day 12 to day 16 of gestation. The cloning of the first observable rearrangements comprised a true "nascent" repertoire (the repertoire derived solely from the ability to rearrange). Comparison of this nascent repertoire with one derived slightly later in development allowed us to assess the degree to which the "nascent" repertoire is shaped in its maturation to an "available" repertoire (prior to challenge with foreign antigen). We observed that κ light chain rearrangement is initiated at approximately day 14 of gestation. The frequency of κ light chain rearrangements increased 5-10 fold/day of gestation. Surprisingly, the frequency of in-frame κ light chain rearrangement also increased on successive days of gestation. Day 14 rearrangements are 34% in frame, while by day 16 rearrangements are 46% in frame, indicating rapid and extensive cellular selection. Lastly, the repertoire is highly skewed. The proportion of Jκ5 rearrangements to Jκ2 and Jκ4 is approximately two fold higher than what is expressed in adult spleen, and the frequency of rearrangement of Vκ4 family members to Jκ5 is much higher than is found to other Jκs, or in the adult spleen.

**FZ 218** Vk HYPERVARIABLE REGION NUCLEOTIDE SEQUENCE IS SPECIFICALLY LINKED TO ALLOTYPE IN b4/b9 HETEROZYGOUS RABBITS. Carl Ritter and Ben Wolf, Departments of Animal Biology and Pathobiology, University of Pennsylvania, School of Veterinary Medicine, Philadelphia, PA 19104.

Two rabbits, serotyped as b4/b9, were infected with T. brucei for 4 weeks. mRNAs were extracted from either spleen (rabbit 8603-A-101) or lymph node (rabbit 8603-A4) and used to prepare cDNA by RT-PCR. PCR products were cloned, sequenced, and analyzed. Each IgK sequence was judged functional by continuity, canonical nucleotides and physical structure of the resulting antibody. From 7 b4 and 2 b9 IgKs, Vk cDNA sequences were analyzed after being co-aligned with the sequence of a germline Vk gene (Lieberman et al., J. Immunol. 133:2753, 1984). Removal of nucleotides from the co-aligned cDNAs common to both germline and cDNAs leaves a map in which the cDNA nucleotides are predominantly (>90%) in columns, indicating reproducible in their placement. 58% of the nucleotide substitutions are in CDR-1, 2 and 3, while 21% are in FR-1. The combined Vk regions have 7 different CDR-1s, 3 different CDR-2s and 8 different CDR-3s. Three additional b9-linked imperfect partial Vk cDNA sequences were obtained, appear to be undergoing partial 5'-nucleotidase destruction, as judged by the incremental 5' shortening. Stepwise destruction of imperfect b9s may be a part of allotype exclusion, leaving 7 b4s and 2 b9s functional. cDNA Vk nucleotide sites were found to fall into four categories, those found only in b4-linked Vk, only in b9-linked Vk, and in those common to both (18 of 58, b9 unique; 52 of 95, b4 unique, 25 common to both b4 and b9 with the same replacements, and 15 common to both with different replacements); Vk nucleotide sites are under allotypic control. Further study will aid in determining whether the Vk cDNA variation in nucleotide sites represents mRNA from B cells before or after antigen activation. (NSF DCB 9005021.)

**FZ 219** DEVELOPMENTAL AND STRAIN DIFFERENCE IN $V_H81X$ GENE EXPRESSION, Dan H. Schulze and Helen Chen, Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD 21201

The mouse immunoglobulin heavy chain variable gene, $V_H81x$, is a member of the $V_H7183$ family which has been demonstrated to be preferentially rearranged in the B cell lines of fetal origin. In this study we examined the regulation of $V_H81x$ gene expression in development and among different strains of mice. Nucleotide sequence analysis of all known BALB/c $V_H7183$ members reveals a unique restriction site (EcoRI) present only in the $V_H81x$ gene, which permits identification of this gene from other 7183 members. cDNAs were prepared from fetal or new born livers and adult spleens. All expressed $V_H7183$ genes were amplified by PCR using a 7183-family-specific primer and a $C\mu$ primer. Following EcoRI digestion of the PCR product and Southern blotting, the filter was hybridized with a labeled oligonucleotide that recognizes an internal consensus sequence common to all 7183 genes. The proportion of the digestable 7183 sequence ($V_H81X$) was determined by autoradiographically. In BALB/c mice the developmental profile of the $V_H81x$ gene expression showed a sequential decrease from fetal to adult stages: the $V_H81X$ message was 3 times more in 14-day fetal liver as compared to 17-day fetus and newborns; and the level was undetectable in adult spleens. Additionally, using a similar strategy for PCR-amplified germline 7183 genes (by a 7183-specific primer and a consensus sequence 3' to every $V_H$ gene [heptamer-nonamer]), we have estimated that in the BABL/c germline, the $V_H81x$ gene represents about 5% of the 7183 family. Similar developmental pattern of this gene expression was also observed in other strains examined, including NZB and Palmerston North (PN). As a comparison, expression of another 7183 member, $V_HV4$ which contains a different restriction site (BsmA2) was measured in context of development in BABL/c. The result showed a relatively constant level of expression throughout different stages.

**FZ 220** STAPHYLOCOCCAL PROTEIN A IS A $V_H3$ RESTRICTED UNCONVENTIONAL ANTIGEN BOUND BY A LARGE PROPORTION OF HUMAN B CELLS. G. J. Silverman,* S. B. Wormsley and M. Sasano*, *University of California, San Diego, La Jolla, CA 92093, Cytometry Associates, Inc. San Diego, CA. 92121

Recent studies have characterized the attributes of a class of bacterial and viral proteins, termed superantigens, that can be bound by 0.1-20% of lymphocytes. Previously, only superantigens that bind to T cell receptors have been described, but based on immunochemical analyses we have recently suggested that *Staphylococcal protein A* (SpA) has a comparable interaction with B cell Ig receptors. These studies demonstrate that, due to binding to a site separate from the Fc portion of IgG, only Fab with certain $V_H3$ H chains bind the "alternative binding site" of SpA. To extend these studies we have used multiparameter flow cytometry to demonstrate that this site on SpA is bound by the surface Ig on greater than 15% of human B cells. SpA binding is also independent of light chain isotype. Significantly, a greater proportion of IgM than IgG bearing cells bound this site on SpA. Sequence analysis of Fab that bind SpA, isolated from a surface display phage, combinatorial expression library, corroborates that many $V_H3$ genes can encode for SpA binding. Immunohistochemical analyses of lymphoid tissue revealed that B cells that bind this site on SpA are common in mantle zones and germinal centers of most follicles. Collectively, these studies raise the possibility that SpA represents a superantigen which may be capable of significantly influencing the B cell repertoire.

**FZ 221** THE INITIATION BUT NOT THE MAINTENANCE OF AN IMMUNE RESPONSE MAY BE INFLUENCED BY THE GLYCOSYLATION OR ENZYMATIC ACTIVITY OF THE ANTIGEN. Mark Suter, Swiss Institute for Allergy and Asthma Research, 7270 Davos, Switzerland.

In man, bee venom phospholipase A2 (BV-PLA2) has both IgE - associated allergenic or IgG - mediated antigenic properties. Human monoclonal or polyclonal antibodies of IgE or IgG recognize restricted discontinuous epitopes involving distinct ε-amino groups of lysine. Native, glycosylated or enzymatically deglycosylated BV-PLA2 was equally effective in inducing type I skin reaction in allergic individuals when compared to recombinant, enzymatically active or singly point mutated, enzymatically inactive BV-PLA2.

In mice, the maximal induction of IgE required ng doses of native BV-PLA2; the maximal induction of IgG required 10 times higher doses of antigen. Recombinant, enzymatically active BV-PLA2 induced similar amounts of antigen - specific IgG but little IgE when compared to native, glycosylated BV-PLA2. Ten times higher doses of recombinant, enzymatically inactive BV-PLA2 were required to obtain comparable IgG responses to native or recombinant enzymatically active BV-PLA2. Recombinant, enzymatically inactive BV-PLA2 induced little if any IgE at any antigen dose used.

The data show that the maintenance of the allergic immune response to BV-PLA2 in man is not affected by the glycosylation or enzymatic activity of the allergen. In contrast, the induction of the immune response and the immunoglobulin isotype induced in mice is influenced by the dose, the glycosylation and by the enzymatic activity of BV-PLA2.

**FZ 222** A HUMAN IMMUNOGLOBULIN HEAVY-CHAIN MINILOCUS ENCODING MULTIPLE V, D, J, AND C REGIONS, Lisa D. Taylor, Roshanak Mashayekh, Kay M. Higgins, and Nils Lonberg, GenPharm International, 2375 Garcia Avenue, Mountain View, CA 94043.
We have developed plasmid cloning vectors that have allowed us to clone 4 different human heavy chain variable gene segments, 16 different diversity segments, 6 different joining segments and 2 different constant region isotypes, together with their respective switch regions, on a single plasmid insert. We used the resulting 80 kb fragment to generate transgenic mice that express a diversity of human sequence immunoglobulins. We will present a molecular analysis of the human V-D-J joints that result from recombination within the minilocus during B-cell development in the transgenic animals.

**FZ 223** RESTRICTED IMMUNOGLOBULIN JUNCTIONAL DIVERSITY IN NEONATAL B CELLS RESULTS FROM DEVELOPMENTAL SELECTION RATHER THAN HOMOLOGY-BASED V(D)J JOINING, Craig B. Thompson, Akhilesh Pandey, and Larry W. Tjoelker, Howard Hughes Medical Institute and Departments of Internal Medicine and Microbiology/Immunology, University of Michigan Medical Center, Ann Arbor, MI 48109
The mechanism by which coding ends are joined during immunoglobulin recombination is poorly understood. Recently short sequence similarities (2-6 bp) observed at the ends of certain variable (V), diversity (D), and joining (J) gene segments of immunoglobulins have been correlated with limited junctional diversity observed in coding exons assembled from these elements. However, it is unclear whether these sequence homologies play any direct role in favoring coding joint formation by influencing the V(D)J recombination process. In this report, we demonstrate that coding sequence similarities do not influence the position of coding joints during *V(D)J recombination in vivo*. Instead, during embryonic development, B cells with certain joining products undergo progressive selection. Developmental selection is completed prior to exposure to external antigens and appears to be determined by the amino-acid sequence encoded by the coding joint. We conclude that the nucleotide sequences of the coding regions do not play a major role in directing V(D)J recombination. Instead, we propose that limited immunoglobulin junctional diversity results from prenatal developmental selection of B cells based on the protein sequence of their surface immunoglobulin antigen-binding site. Sequence identities at the ends of coding segments may have evolved because they increase the likelihood that a selectable antigen-binding site is created during a random recombination process.

**FZ 224** PROTEIN SERINE/THREONINE KINASE ACTIVATION BY SURFACE IGM IN APOPTOTIC B LYMPHOCYTES. EVIDENCE FOR AN ALTERNATE AMPLIFICATION LOOP.
Mary A. Valentine* and James Weiel+
*Department of Microbiology, SC-42, University of Washington, Seattle WA 98195 and +Glaxo Inc. Research Triangle Park, NC 28809.
Ligation of the antigen receptor on human B lymphocytes results in the activation of tyrosine kinases whose signals, in turn, are amplified by the involvement of other pathways including several protein serine/threonine kinases. As receptor ligation can results in diverse effects including proliferation, differentiation or death, it would be predicted that diverse signaling pathways should be engaged to specify these alternate events. Ligation of the antigen receptor on the Burkitt's lymphoma cell line, Ramos, results in programmed cell death (apoptosis) of the cells. The protein serine/threonine kinases affected by IgM signaling in Ramos cells differ from those engaged in normal cells whose response to IgM results in proliferation. Ligation of IgM on normal tonsillar B cells activates protein kinase C, and stimulates phosphorylation of several peptides that serve as substrates for the *erk* (aka MjAP-2), *rsk* (aka S6 kinases), and calcium/calmodulin-dependent (CaM-KII) kinases. In contrast, IgM negatively regulates CaM-KII, does not engage PKC, but does involve early and prolonged activation the the 70 kDa *rsk*. The necessary role of these various kinases to initiation of programmed cell death in the Ramos cells will be discussed.

**FZ 225** MOLECULAR EVOLUTION OF THE HUMAN IGE RESPONSE: HIGH INCIDENCE OF SHARED MUTATIONS AND CLONAL RELATEDNESS AMONG ε $V_H5$ TRANSCRIPTS FROM THREE ATOPIC DERMATITIS PATIENTS, Nienke van der Stoep, Joke van der Linden and Ton Logtenberg, Department of Immunology, University Hospital Utrecht, Utrecht, The Netherlands.
Atopic Dermatitis is an inflammatory skin disorder characterized by a chronically relapsing course. Although the primary cause of atopic dermatitis is unknown, substantial evidence suggests that the exessive production of IgE antibodies, reactive with a wide variety of environmental allergens, likely contributes to the pathogenesis of this disorder. The role of allergen reactive T cells in controlling IgE class switching is beginning to be elucidated, however little is known about the B lymphocytes that are recruited into the IgE response. In the present study we have employed the cDNA/PCR approach to analyze the molecular structure of V regions expressed in the IgE antibodies. Our data show that members of the $V_H3$-$V_H6$ gene families may be utilized. The small human VH5 gene family was abundantly expressed. Comparison of the nucleotide sequences of 19 ε $V_H5$ transcripts with the germline $V_H5$ genes of three atopic dermatitis patients revealed the accumulation of high numbers of somatic mutations. A surprisingly high proportion of replacement and silent mutations was shared among sequences from the three unrelated individuals. In two patients we detected clonally related but diverged transcripts, permitting the construction of a genealogical tree in one patient.

FZ 226 MECHANISMS OF NEONATAL B CELL UNRESPONSIVENESS TO ANTIGEN

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Although much of immunological development occurs *in utero*, most mammals do not achieve full immunocompetence until several days after birth. Thus, B lymphocytes from neonatal mice, unlike those from adults, do not proliferate or secrete antibodies in response to antigenic stimulation. While the biochemical basis for neonatal B cell unresponsiveness is not fully understood, previous studies in our laboratory have suggested that it may involve uncoupling of the B cell antigen receptor (sIgM) from the phosphatidylinositol (PI) signalling pathway. To determine the importance of this defect, and to further explore the mechanisms of neonatal B cell unresponsiveness, we have studied reagents that modulate neonatal B cell responses to sIgM crosslinking. We now show that treatment of neonatal cells with bacterial lipopolysaccharide (LPS) allows them to proliferate to subsequent stimulation by anti-IgM antibodies, probably by recoupling the receptor to the PI pathway. We also demonstrate that T cell clones, and the T cell-derived lymphokine interleukin-4 (IL-4), are capable of synergizing with anti-IgM to induce neonatal B cell proliferation. In contrast to LPS, IL-4 must be present during anti-IgM stimulation, and does not act by recoupling the receptor to the PI pathway. The mechanism of this effect is currently under investigation, and will likely provide new insight into the signalling pathways involved in neonatal B cell unresponsiveness. In addition to these functional studies, we have begun to study the physical and phenotypic characteristics that distinguish neonatal cells from mature, antigen-responsive ones. Based on these studies, we hope to determine whether induction of responsiveness by LPS and IL-4 is accompanied by a shift to a more mature phenotype.

FZ 227 ANALYSIS OF IMMUNOGLOBULIN LIGHT CHAIN REPERTOIRE USED IN ANTIGEN SPECIFIC HYBRIDOMAS FROM TRANSGENIC MICE

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We have set up a transgenic mouse model to analyze the contribution of heavy chain vs. light chain to antigen-specific binding. In this model, we introduced the heavy chain of L6, an antibody (Ab) against a human tumor associated antigen. Random light chains from the mouse genome that joined with the L6 heavy chain were identified. The contribution of light chains to Ab affinity and cross-reactivity were compared.

The heavy chain transgene was constructed with the VDJ region from L6 placed adjacent to genomic Cu sequences. Transgenic mice with single or multiple (>10) transgene copies were immunized with the L6 antigen. Hybridomas from these mice were analyzed for antigen specificity, affinity, and transgene vs endogenous mu expression.

PCR analysis of the light chains in L6 antigen specific hybridomas identified the light chain families that could associate with the heavy chain transgene and contribute to antigen specificity. The same Vk family and J5 were used in all of these Abs. This was the same light chain family used in the original L6 Ab. The hybridoma light chains are being cloned and re-expressed in cell lines with the L6 IgM transgene to determine the contribution of light chain somatic mutations to Ab affinity.

This analysis suggests that there is a single light chain family that contributes to L6 antigen specificity with the given heavy chain. Detailed sequence analysis will be used to identify amino acids affecting antigen binding.

FZ 228 MOLECULAR GENETIC DIVERSIFICATION OF VARIABLE REGION GENES FROM GALT B CELLS RESPONDING TO ENVIRONMENT ANTIGENS IN THE TWO PRIMARY GERMINAL CENTER COMPARTMENTS, P.D. Weinstein*, A.O. Anderson° and R.G. Mage*, *LI/NIAID, NIH, Bethesda, MD 20892; °DAD/USAMRIID, Ft. Detrick, Frederick, MD 21702

Alterations in the germline variable region DNA sequence are believed to occur by either somatic mutation or gene conversion in B cell follicular regions known as germinal centers. These follicles form in response to T-dependent antigens, and in gut associated lymphoid tissue (GALT) are constitutive due to the continual stimulation provided by environmental antigens. Germinal centers can be subdivided into two distinct regions known as the dark zone and the light zone, which contain B cells known respectively as centroblasts and centrocytes. We have been able to show that each of these germinal center populations has distinct staining patterns with various lectins allowing us to use a micromanipulator to isolate appendix germinal center B cells from each of these zones. We have found that GALT germinal centers appear to form from only a few precursor B cells, and that the genomic DNA variable region sequence was altered in both centroblast and centrocyte populations, with the centrocyte population containing more base pair changes than centroblasts. These results lead us to believe that even though GALT germinal centers are present continually, due to the oligoclonality of the response, each germinal center may be responding to a single antigen. In addition, it appears that the processes used to alter the genomic variable region DNA sequence may be ongoing during germinal center B cell development, and not restricted to a particular point in the maturation process.

FZ 229 THE ROLE OF SELECTION IN THE DEVELOPMENT OF THE B CELL REPERTOIRE, Elizabeth A. Whitcomb and Peter H. Brodeur, Program in Immunology and Department of Pathology, Tufts University Sackler School of Graduate Biomedical Sciences, Boston, MA 02111

We have used $V_\kappa$ cDNA libraries to determine the utilization frequency of individual $V_\kappa$ exons in adult mouse B cells. We have identified two functional $V_\kappa$ exons which are expressed at very different frequencies. Whereas $V_\kappa$1-A encodes 9% of the cloned $V_\kappa$ cDNAs, the $V_\kappa$22 gene encodes only 0.2% of the same $V_\kappa$ cDNA library. To ascertain whether this difference reflects ligand-mediated selection via the B cell antigen receptor, we are determining the ratio of productive to non-productive rearrangements of these two $V_\kappa$ exons in a population of mature B cells. Our initial results reveal that the $V_\kappa$1-A gene is joined in-frame in 85% (23 of 27) of the rearrangements sequenced. A set of rearrangements utilizing the underrepresented $V_\kappa$22 gene contained only 60% productive rearrangements (6 of 10). Recently Milstein et.al. (E.J.I. 1992.22:1627) reported 50% in-frame rearrangements in a set of $V_\kappa$4 rearrangements. We have found that while the $V_\kappa$4 family rearranges at a high frequency in adult bone marrow pre-B cells, it is not overexpressed in the adult splenic B cell repertoire. Taken together these results indicate a significant role of selection in shaping the pre-immune B cell repertoire.

*Cellular and Molecular Aspects of the Microenvironment*

**FZ 230** The Regulatory Role of Human Fetal Bone Marrow Stromal Cell Lines on B Lymphopoiesis, L.G. Billips, A.K. Stankovic, L. Gartland, and M.D. Cooper, Department of Developmental & Clinical Immunology, University of Alabama-Birmingham, Birmingham, AL 35294.

B lymphocytes are a population of rapidly-renewed cells in the bone marrow of mammals. Although a number of cell types appear to play a role in providing positive and negative signals which maintain B lymphopoiesis, the essential component for B cell development in vitro appears to be a population of adherent stromal cells of uncertain lineage. In order to better define the complex relationship between B lymphocyte precursors and their regulatory microenvironment, we isolated nontransformed cloned stromal cell lines from human fetal bone marrow. These stromal cell lines were cloned by limiting dilution and have been passaged in culture for >10 months. In vitro assays involved culturing these stromal cell lines with $CD19^-sIgM^-$, $CD20^-sIgM^-$, or $CD34^+$ sorted fetal bone marrow cells. One of the cell lines, HBM-W2, supported the development of $IgM^+$ B cells from all of these sorted bone marrow populations. Another stromal cell line, HBM-D3, did not support sIgM expression by $CD19^-sIgM^-$ bone marrow cells. Culturing of HBM-D3 stromal cells with $CD20^-sIgM^-$ cells did result in sIgM expression, but a higher percentage of B cells was consistently found when the HBM-W2 stromal cell line was used. Comparison of two stromal cell lines which differ in their support capacities will provide for a better understanding of the regulatory requirements involved in human B lymphopoiesis. (Study funded by NIH grant #CA09128)

**FZ 231** INTERLEUKIN-10 ENHANCES B-LYMPHOPOIESIS IN THE BONE MARROW, Jay S. Fine, Heather D. Macosko, Michael J. Grace and Satwant K. Narula, Department of Cell Biology, Schering-Plough Research Institute, Bloomfield, NJ 07003.

The effect of in vivo and in vitro interleukin-10 (IL-10, cytokine synthesis inhibitory factor) treatment on murine B lymphocyte development in the bone marrow was determined. Young adult DBA/2 female mice were administered 1 μg mIL-10 daily by intraperitoneal injection and bone marrow, spleen and peripheral blood cells examined by multiparameter flow cytometry at various times. By two days after the initiation of IL-10 dosing, there was an increase in the percentage and number of marrow $B220^+$ $sIgM^-$ pre-B cells in treated mice compared to vehicle-dosed control animals. By day 6, we observed an enlargement of both the pre-B and $B220^+$ $sIgM^+$ mature B cell population in bone marrow, as well as a significant increase in the number of marrow CFU-pre-B, from IL-10-treated mice. By day 10 however, no differences in B lymphocyte development were detected, despite continued daily administration of IL-10, implying that compensatory mechanisms may develop in animals treated with this cytokine. Only minor differences in splenic or peripheral blood B and T cell populations were noted during these times. Interestingly, IL-10 appeared to synergize in a dose-related manner with IL-7 in CFU-pre-B cultures of normal unfractionated bone marrow cells. This effect of IL-10 was specifically blocked by the addition of anti-IL-10 antibodies. These data suggest that IL-10 may directly influence murine B-lymphopoiesis.

**FZ 232** ENRICHMENT OF PRIMITIVE HEMATOPOIETIC PRECURSORS IN CLOSE ASSOCIATION WITH NATIVE BONE MARROW STROMAL CELLS, Phillip E. Funk[1], Paul W. Kincade[2], and Pamela L. Witte[1,3], Depts.of Microbiology[1] and Cell Biology[1,3], Loyola Univ. Med. Cntr., Maywood, IL 60153, and [2]Oklahoma Med. Rsch. Found., Oklahoma City, OK 73104

Bone marrow stromal cells are an integral part of the hematopoietic microenvironment, providing both cell contact and soluble growth factor signals to developing hemopoietic cells. Stromal cells studied as cell clones or lines after repeated passage in culture are heterogeneous, but it is unclear if this represents heterogeneity within the normal stromal cells, outgrowth of rare cells in culture, or adaptation of the cells to various culture conditions. Our goal is to enrich stromal cells directly from bone marrow cell suspensions. We have previously reported that stromal cells are enriched in cell aggregates present in murine bone marrow cell suspensions. These aggregates exhibit a reticular staining pattern for VCAM1 expression, mimicking that seen in marrow sections, and i. v. injection of anti-VCAM1 mAb M/K2 disrupts the aggregates, implicating the VCAM1 molecule as an adhesive mechanism holding aggregates together. This also suggests that aggregates occur naturally within the marrow and preserve areas of native marrow architecture. Isolated aggregates are able to form both Whitlock and Dexter type long-term bone marrow cultures from one-half to one-fifth as many initiating cells. Using the appearance of areas of proliferating hematopoietic cells (cobblestone areas) on pre-formed stromal layers as a means to detect primitive hematopoietic progenitors we show that aggregates are also enriched in long term culture initiating cells. Thus, aggregates are enriched in both stromal cells and primitive hematopoietic progenitors and these are maintained in close physical proximity at least in part by adhesion using VCAM1. Aggregates are not enriched in cells responsive to WEHI 3 conditioned medium (IL-3), SCF, or IL-3 plus SCF in soft agar colony assays. Preliminary data indicates that stromal cells may be effectively sorted from collagenase dispersed aggregates based on forward versus 90° light scatter. Further sorting based on expression of VCAM1 may yield further stromal cell enrichment. (Supported by American Cancer Society grant IM-612)

**FZ 233** REGULATION OF PRO-B CELL PROLIFERATION BY IL-7, IGF-1, AND *CKIT*-LIGAND. Laura F. Gibson, Debra Piktel, and Kenneth S. Landreth, Departments of Pediatrics, Microbiology and Immunology, and the Mary Babb Randolph Cancer Center, West Virginia University Health Sciences Center, Morgantown, WV 26506.

Pro-B cells from mid gestation fetal liver have been shown to have extensive proliferative potential in the presence of an adherent layer of bone marrow stromal cells and IL-7. We established a panel of murine pro-B cell lines from primary cultures of 12-14 day fetal liver cells by repeated passage and cloning at limiting dilution in the presence of a cloned stromal cell line and 50 units/ml rIL-7. Resulting cell lines either had $DJ_H$ or no detectable rearrangement of the Ig heavy chain gene complex and none of these pro-B cell clones had detectable light chain gene rearrangements. None of these cells expressed CD45(B220) or $c\mu$. Pro-B cell lines proliferated in the presence of IL-7 when removed from stromal cell layers. Both IGF-1 and *ckit*-ligand (KL) potentiated this proliferative response to IL-7 with equivalent efficiency. Bone marrow stromal cell lines used in this study have detectable mRNA for both IGF-1 and KL. Although the effect of IL-7 on pro-B cell proliferative responses was potentiated by IGF-2, no transcripts for IGF-2 or IGF-2-receptor were found in either stromal cells or pro-B cell lines. These studies suggest that the essential cytokine for expansion of pro-B cells in vivo is IL-7. IGF-1 and KL are redundant potentiating signals for IL-7 mediated pro-B cell proliferation and this suggests that failure to express either of these cytokines should not, of itself, result in failure of the developing B lineage. These results provide an explanation for normal B cell production in mice with defective *ckit* expression or those treated with antibodies to *ckit*. (Supported by PHS grant AI-23950 and Hoffmann-LaRoche, Inc. LFG is the recipient of a fellowship grant from the Cancer Research Institute.)

FZ 234 CYTOKINE REGULATION OF FETAL LIVER B CELL PROGENITOR DEVELOPMENT, Brian Gliniak, Ian McAlister, Fred Ramsdell, Ken Brasel and Douglas Williams, Department of Experimental Hematology and Immunology, Immunex Corporation, 51 University St., Seattle, WA 98101

We have established a Whitlock/Witte type culture system using day 15 murine fetal liver cells to study the role of specific cytokines in B cell progenitor development. The nonadherent cells from these cultures appear to quickly become committed to the lymphoid lineage as judged by the appearance of predominantly (>80%) B220+ cells, their ability to reconstitute thymic lobe cultures, and their inability to form CFU-S. The remaining B220- cells appear to contain the lymphoid progenitor cells. Isolation of this population by cell sorting and addition back to fresh stromal layers quickly results in the loss of B220- cells and the generation of B220+ cells. IL-7 is absolutely required to initiate and sustain these cultures. The B220- population shows a strong synergistic proliferative response to IL-7, MGF, and IGF-1, whereas the B220+ population does not. Supernatants derived from primary day 13 fetal liver stroma contains an activity capable of inhibiting the synergistic action of IL-7 and MGF. This activity could be reproduced with TGF-ß and eliminated with TGF-ß neutralizing antibodies. Therefore, fetal liver B cell progenitor development appears to require the coordinated expression of both stimulatory and inhibitory cytokines.

FZ 235 INHIBITION OF MURINE B CELL DEVELOPMENT IN VIVO BY AN ANTI-IL7 ANTIBODY

Kenneth Grabstein*, Thomas Waldschmidt**, Anthony Namen*, Alan Alpert*, Bruce Hess*, Norman Boiani*, Frederick Finkelman***, Philip Morrissey* *Immunex Corp., Seattle USA **University of Iowa, Iowa City USA ***Uniformed Services University of the Health Sciences, Bethesda USA

IL7 has been considered to have an important role in the development of B lymphocytes based on its ability to stimulate the proliferation of pre-B cells in vitro. More recently, it was shown that in mice receiving IL7 treatment, or in IL7 transgenic mice, significant stimulation of B lymphopoiesis occurred. A murine anti-human IL7 monoclonal antibody was generated and found to neutralize both murine and human IL7 activity in vitro. Treatment of mice with this antibody results in the cessation of B cell development in the marrow. No effect was observed on the earliest B lineage cells detectable, which express the CD45R, but not the BP-1 cell surface markers. In spite of the marked reduction in primary B lymphopoiesis, very little effect was seen among the peripheral B cell populations over a course of treatment of up to 6 weeks. We conclude that IL7 is an essential mediator of B cell development in mice. Our results further suggest that the majority of peripheral B cells are not rapidly turning over.

FZ 236 INTERFERON-γ ARRESTS PROLIFERATION AND INDUCES APOPTOSIS IN STROMAL CELL AND IL-7 DEPENDENT NORMAL PRE-B CELL LINES AND CLONES, BUT DOES NOT LEAD TO DIFFERENTIATION TO SURFACE Ig-POSITIVE B CELLS, Ulf Grawunder, Fritz Melchers, Antonius Rolink, Basel Institute for Immunology, CH-4005 Basel, Switzerland

B cell precursors (preB cells) can be cultured *in vitro* in the presence of interleukin-7 (IL-7) and bone-marrow derived, adherent stromal cells for extended periods of time. These cells express the preB cell specific genes $\lambda_5$ and $V_{preB}$ and are $D_HJ_H$-rearranged on both chromosomes, while the light-chain loci (κ and λ) remain in germline configuration. The cells can be differentiated to sIg-positive B cells after removal of IL-7, a process, which is accompanied by apoptosis.

Since previous studies have shown that interferon-γ (IFN-γ) is able to induce sIg-expression in the preB lymphoma line 70Z/3, we investigated the influence of this cytokine on normal preB cell lines. IFN-γ (as low as 0.1-1U/ml) inhibited proliferation and induced apoptosis in the continued presence of stromal cells and IL-7. Under these conditions the preB cells did not differentiate to sIg-positive B cells. In bcl-2 transgenic preB cells, IFN-γ inhibited proliferation, did not induce differentiation, but no apoptosis was observed. These results are discussed in view of a possible role of IFN-γ in B cell differentiation at the stage of transition from preB to B cells.

FZ 237 SYNERGISTIC INTERACTION BETWEEN INTERLEUKIN-12 (NATURAL KILLER CELL STIMULATORY FACTOR, CYTOTOXIC LYMPHOCYTE MATURATION FACTOR) AND STEEL FACTOR IN SUPPORT OF PROLIFERATION OF MURINE LYMPHOHEMOPOIETIC PROGENITORS IN CULTURE, Fumiya Hirayama*, Naoyuki Katayama*, Steven Neben†, Debra Donaldson†, Elliot B. Nickbarg†, Steven C. Clark† and Makio Ogawa*, *Department of Medicine, Medical University of South Carolina and *Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC 29403 and †Genetics Institute, Cambridge, MA 02140

Interleukin 12 (IL-12) was independently identified as a natural killer cell stimulatory factor and as a cytotoxic lymphocyte maturation factor. It is a heterodimer consisting of 40kDa (p40) and 35kDa (p35) proteins. Recently, studies revealed amino acid sequence homology between p40 and the extracellular domain of IL-6 receptor and among p35, IL-6 and G-CSF. We have reported earlier that IL-6 and G-CSF are synergistic factors for initiation of cell cycling of primitive progenitors. Therefore, we have investigated the effects of IL-12 on proliferation of murine myeloid and lymphohemopoietic progenitors in methylcellulose cultures. Whereas IL-12 and steel factor (SF) alone did not effectively support multilineage myeloid colony formation by mononuclear marrow cells of normal mice, the combination of the two yielded significant numbers of such colonies. When tested on highly enriched marrow cells from mice treated with high-dose 5-fluorouracil, the combination of IL-12 and SF, but not the single factors, was effective in support of formation of various colony types. Approximately 25% of these colonies yielded pre-B cell colonies when replated in secondary culture in the presence of SF and IL-7, indicating the IL-12, like IL-6, IL-11 and G-CSF can interact with SF in supporting the development of primitive lymphohemopoietic progenitors. Interestingly, as noted with the other cytokines, combinations of IL-12 with IL-3 yielded colonies which possess myeloid cells but lack B-lymphoid potential. These results demonstrate that IL-12, a cytokine previously believed to be involved in the activation of mature T cell and natural killer cell populations, also has the potential for growth regulation with various hematopoietic cells, including committed myeloid and primitive lymphohemopoietic progenitors.

**FZ 238** ANALYSIS OF THE GROWTH FACTOR REQUIREMENTS OF B CELL PROGENITORS FROM FETAL LIVER AT DAY 12 OF GESTATION. Barbara L. Kee, Ana Cumano* and Christopher J. Paige. The Wellesley Hospital Research Institute, Toronto, Canada and Institute Pasteur, Paris, France*.

We have investigated the ability of 3 stromal cell derived cytokines, interleukin 7 (IL7), IL11 and mast cell growth factor (MGF), to support the growth and differentiation of B cell progenitors present in fetal liver (FL) at day 12 of gestation. B cell progenitors were enriched by panning, on antibody coated petri dishes, for cells expressing AA4.1 but not B220 or Mac-1. Limiting dilution analysis revealed that there are 600-2000 precursors/FL with this phenotype which can develop into IL7 responsive cells in the presence of the fibroblastic cell lines, S17 or NIH 3T3. These IL7 responsive cells can develop into immunoglobulin (Ig) secreting B cells in the presence of stromal cells and lipopolysaccharide (LPS). To further define the growth factors which support early B cell development we substituted stromal cells with IL7, MGF or IL11. We found that IL7, MGF or IL11 alone or the combination of IL11 + IL7 fail to support the differentiation of AA4.1$^+$B220$^-$Mac-1$^-$ FL cells into IL7 responsive pre-B lymphocytes. However, 150-200 progenitors/FL could differentiate in the presence of MGF + IL7. IL-11 in combination with MGF + IL7 increased the frequency of responding cells approximately 2 fold. The majority of progenitors grown under these conditions are uncommitted for the expression of heavy chain allotypes. These data document defined growth conditions which allow primitive Ig-unrestricted B cell progenitors to differentiate to IL7 responsive pre-B lymphocytes. We have previously shown that Ly6$^+$ progenitors present in this population are uncommitted to the B lineage differentiation pathway (Cumano, A. et al, Nature 356:612, 1992), giving rise to both B cells and macrophages. The influence of these growth factors on lineage uncommitted progenitors is currently under investigation. The role of MGF in stromal cell support of B cell progenitors growth is being analyzed further by the use of neutralizing antibody.

**FZ 239** EFFECT OF RETINOIC ACID ON IL6-DEPENDENT DIFFERENTIATION OF BLOOD B CELLS FROM PATIENTS WITH IgM GAMMAPATHY, Yves Levy and Jean-Claude Brouet, Laboratory of Immunopathology, Research Institute on Blood Diseases, Hôpital Saint-Louis, 75475 Paris cedex 10, France.

We have previously reported that clonal blood B cells from patients with IgM gammapathy spontaneously differentiate to plasma cells in vitro by an IL6 autocrine dependent pathway. Here we investigated the effects of all-trans retinoic acid (ATRA) on this differentiation process. Seven patients with IgM gammapathy were studied. Purified blood B cells were obtained after depletion of monocytes by L-leucine methyl ester treatment or adherence and of T cells by E-rosetting. Cells were cultured for 7 days in medium alone or in the presence of ATRA ($10^{-5}$ M). In all cases, B cells cultured in medium alone spontaneously differentiated to plasma cells with IgM secretion (mean : 1400 ng/ml ; range 110-3000 ng/ml). Biologically active IL6 was found in all supernatants collected at day 2 (mean 70 pg/ml ; range 5-150 pg/ml). In the same culture conditions, control normal B cells did not differentiate or produce IL6. In the presence of ATRA, the differentiation process was inhibited (50-80% decrease in IgM secretion) in four cases with benign IgM gammapathy whereas no effect (less 25% decrease in IgM secretion) was noted in 3 patients with Waldenström's macroglobulinemia. The inhibition was correlated with a reduction of IL6 concentration in supernatants (mean 81% ; range 50-100%). In contrast, IL6 production was unaffected in the 3 cases where ATRA had no effect. Blood B cells from these patients featured membrane IL6 receptors ; preliminary data indicate that IL6 receptor expression was down regulated by ATRA in some cases. These results provide evidence that the differentiation process of B cells from patients with monoclonal IgM gammapathy differ according to the presence of an overt lymphoid malignancy as suggested previously upon the differential effect of neutralizing anti-IL6 antibodies.

**FZ 240** INTERLEUKIN-7 (IL-7) STIMULATES PROLIFERATION OF TDT-POSITIVE AND TDT-NEGATIVE PRO-B CELLS FROM RAT BONE MARROW IN VITRO
Sean D. McKenna and Irving Goldschneider, Department of Pathology, School of Medicine, University of Connecticut Health Center, Farmington, CT 06030

The stromal cell-derived cytokine, IL-7, has been identified as a potent stimulator of murine cμ$^+$ pre-B cells. However, reports on the role of this cytokine in stimulating less mature B-lineage cells have been conflicting. Using a novel xenogeneic cell culture system which selectively supports the growth of apparently normal TdT$^+$ lymphoid precursor cells and their TdT$^-$ precursors from rat BM, the responsiveness of these primitive B-lineage cells to murine rIL-7 was examined directly. The results demonstrate that rat TdT$^+$ lymphoid cells could be maintained in short-term culture with IL-7-supplemented medium but not with control medium alone. Characterization of the lymphoid cells that are maintained by IL-7 revealed them to include both HIS24$^+$/HIS50$^-$/cμ$^-$ and HIS24$^+$/HIS50$^+$/cμ$^-$ phenotypic subsets, characteristic of early and late pro-B cells, respectively. However, these cells were not induced to differentiate into cμ$^+$ pre-B cells. Significant thymidine incorporation was observed in culture-generated lymphoid cells upon treatment with IL-7, and this proliferative response was inhibited by addition of neutralizing antibody to IL-7. These results were confirmed by BrdU incorporation studies, which demonstrated that IL-7 selectively induced the proliferation of HIS24$^+$/HIS50$^-$/TdT$^-$ and HIS24$^+$/HIS50$^-$/TdT$^+$ B-lineage stem/progenitor cells. Taken together, these findings demonstrate that, in the rat at least, IL-7 can stimulate the survival and proliferation of B-lineage cells at the earliest stages of lymphoid development. Inasmuch as these pro-B cells were not induced by IL-7 to differentiate into cμ$^+$ pre-B cells, the results further suggest that IL-7 may serve to selectively stimulate the proliferation of early B-lineage cells at multiple defined stages of development, including both early pro-B cells and early pre-B cells. (Supported in part by ACS Grant IM-645 and NIH Grant 32752.)

**FZ 241** FUNCTIONAL DISTINCTIONS BETWEEN STEM CELL FACTOR AND A LOW MOLECULAR WEIGHT LYMPHOPOIETIC COFACTOR, Michael J. Muirhead and Thomas J. Waldschmidt, McClellan VA Medical Center, Little Rock, AR 72205 and University of Iowa College of Medicine, Iowa City, IA 52242

We have previously reported that the ultrafiltrate of 3E stromal cell conditioned medium (CM) synergizes with an optimal dose of Interleukin 7 (IL-7) in support of normal pre-B cell growth and of an IL-7 dependent pre-B cell clone (Witte-Whitlock clone 3). Stem Cell Factor (SCF) also synergizes with IL-7 and it has been claimed that SCF accounts for most of the non-IL-7 growth promoting effects of stromal cell CM on B lymphoid progenitors. In contrast, we find that recombinant SCF, unlike 3E CM, has no direct or synergizing activity on clone 3 cells. The ultrafiltrate of 3E CM, in contrast to SCF, has no activity on CFU-GM when used in concert with GM-CSF in a myeloid colony assay. In addition, the early phase of long-term Witte-Whitlock (WW) mouse bone marrow cultures contains predominantly BP-1 positive, S7 negative pre-B cells which are modestly responsive (2 fold growth in short term cultures) to IL-7 alone, and this is enhanced by either SCF or the ultrafiltrate of 3E CM. In contrast, the late phase of WW cultures contains almost exclusively BP-1 negative pre-B cells which are S7+/-. These cells respond dramatically (10-fold growth) to IL-7 alone but are not further boosted by the addition of SCF. Nevertheless, these primitive pre-B cells respond in synergistic fashion to optimal doses of IL-7 and the ultrafiltrate of 3E CM; and SCF further synergizes with this combination (12-15 fold growth). Thus, there appear to be at least two non-IL-7 lymphopoietic cofactors with differential proliferative effects on pre-B cell subsets.

**FZ 242** SYNERGISTIC EFFECT OF RECOMBINANT HUMAN STEM CELL FACTOR (SCF) AND LEUKEMIA INHIBITORY FACTOR (LIF) ON THE PROLIFERATIVE RESPONSE OF B ACUTE LYMPHOBLASTIC LEUKEMIA (B ALL) CELLS TO INTERLEUKIN 3 (IL3) AND INTERLEUKIN 7 (IL7). Sabine Pontvert-Delucq[1], Claude Baillou[1], Etienne Vilmer[2], Albert Najman[1] and François M. Lemoine[1], [1]Department of hematology, Faculty of medicine Saint Antoine and [2]Hôpital Robert Debré, Paris, France.
The mechanisms regulating the proliferation and the differentiation of B lymphoid precursors are unknown because of the lack of specific assays. However, using neoplastic B cell precursors, it has been recently shown that IL3 and IL7 stimulate their proliferation and that stromal cells can prevent the apoptotic cell death of B ALL cells. In order to investigate the effects of stromal regulatory molecules such as SCF and LIF on B leukemic cells, $10^5$ B ALL cells/well were incubated in 96 wells tissue culture plates in RPMI medium containing 10% fetal calf serum, 1% glutamine, 50 µM 2-mercaptoethanol, 2% antibiotics and various combination of recombinant human (rh) IL7 (1000U/ml), rh IL3 (100U/ml), rh LIF (1000U/ml) and/or rh SCF (100ng/ml) for 7 days. Then, the proliferation was measured by $^3H$ thymidine incorporation. We showed that IL7 or IL3 alone or IL7 + IL3 stimulated the proliferation of B ALL cells by 22±5 fold, 16.5±5 fold and 31±10 fold respectively by comparison to cells alone (no growth factors). Whereas LIF and/or SCF had no effect by themselves , they enhanced the proliferation of B ALL blasts by 50±18.5 fold when added to IL3 and IL7. These results indicate that LIF and SCF exert a synergistic effect on the proliferation of B ALL cells in response to IL7 and IL3 and suggest that these stromal molecules might be involved in the regulation of human B lymphopoiesis.

**FZ 243** IL-7 IS EXPRESSED BY BONE MARROW DERIVED ADHERENT CELLS AND IS REQUIRED FOR *IN VITRO* HUMAN B CELL LYMPHOPOIESIS, Daniel H. Ryan, Bonnie L. Nuccie, Camille N. Abboud, and Jane L. Liesveld, Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, NY 14642.

We have recently described an *in vitro* culture system that supports the growth of colonies of TdT+ early B cell precursors (BCP) from CD34+ human bone marrow cells. Development of BCP colonies requires an adherent layer of bone marrow derived fibroblast-like cells (BM-FB). To investigate the role of IL-7, neutralizing antibody to IL-7 was added at the start of the 14-day culture, causing an 84% reduction in BCP colonies (± 6% SEM; n=7; p<.0001). The specificity of the antibody was supported by the dose-dependent ability of recombinant human IL-7 to reverse most of the inhibitory effect of anti-IL-7. IL-7 mRNA transcripts were identified in unstimulated BM-FB after a single round of PCR (10 ng cDNA amplified for 30 cycles), although levels of IL-7 protein in the BM-FB or supernatant medium were too low to detect by ELISA. Unexpectedly, BCP colonies were inhibited in a dose-dependent fashion (49% ± 14% at 10 ng/ml; n=9; p<.01) by exogenous IL-7 added at the start of culture. Since IL-7 induces monocyte IL-1 expression, and IL-1 is inhibitory to murine BCP, the effect of IL-1 on BCP colonies was examined. IL-1 (200 pg/ml) totally abolished BCP colony formation (n=6). The specificity of this result was confirmed by its complete reversal with neutralizing anti-IL-1 antibody. Although anti-IL-1 by itself did not affect BCP colonies, it reversed the inhibitory effect of 100 ng/ml IL-7, suggesting that exogenous IL-7 may indirectly suppress BCP colonies by inducing IL-1 expression. A possible source of IL-1 is macrophage-like cells resident in the adherent layer or derived from the CD34+ progenitors as identified by surface marker expression. These results suggest that endogenously presented IL-7 supports early B cell lymphopoiesis but that exogenous IL-7 can exert an inhibitory effect, due to increased dose, loss of localization of expression, or differences in microenvironmental presentation of endogenously expressed vs exogenous cytokine.

**FZ 244** *ISOLATION OF HUMAN B CELL PRECURSORS AND THE EFFECT OF TUMOUR NECROSIS FACTOR ON PROLIFERATION.* *Cecilia Skjønsberg, Erlend B. Smeland, Steinar Funderud and Heidi Kiil Blomhoff, Department of Immunology, Institute for Cancer Research, The Norwegian Radium Hospital, 0310 Oslo, Norway.* *A simple and reproducible method for isolation of human B-cell precursors (BCP) from bone marrow aspirates has been developed in our laboratory. B-lineage cells were isolated directly from bone marrow by positive selection through rosetting with magnetic beads (Dynal) coated with the anti-CD19 monoclonal antibody AB1. Detatchment of beads was obtained through over-night incubation. CD22- and sIg-positive cells were removed by negative depletion. Flowcytometric analysis of the cells show that the contamination of non-B lineage cells was <1%. The cells show normal proliferative response in in vitro cultures to Interleukin (IL)-3, IL-7, stem cell factor and LMW BCGF. We and others have shown that Tumour Necrosis Factor (TNF) has an inhibitory effect on the proliferation of leukemic BCPs. We are currently examining the effect of TNF on normal BCPs as well as TNF receptor expression and regulation. These data will be presented.*

**FZ 245** A MONOCLONAL ANTIBODY DISTINGUISHES ALL STROMAL CELLS IN WHITLOCK CULTURES AND A SUBSET OF RETICULAR BONE MARROW CELLS, Pamela L. Witte, Muriel Hergott, Lisa M. Frantsve, Robert P. Stephan, and Phillip E. Funk, Dept. of Cell Biology, Neurobiology, and Anatomy, Loyola Univ. Med. Cnt., Maywood, IL 60153.
Several reports now document the importance of bone marrow and fetal liver stromal cells to B-cell development in vitro. However, the exact nature of the stromal cell remains elusive. With the goal of defining the in vivo source of culturable stromal cells, we immunized Armenian hamsters with FACS-sorted, unpassaged stromal cells from Whitlock cultures. The splenocytes were fused with mouse myeloma P3X63Ag8.653. One resulting mAb, 8.28, distinguished all the large, lymphocyte-binding stromal cells in Whitlock cultures but did not stain macrophages, lymphocytes, or uncultured hemopoietic cells. We examined lymphopoietic cytokine production by the $8.28^+$ cultured stromal cells. At five weeks of culture, these cells appeared to constituitively transcribe IL-7, M-CSF, SCGF, and LIF genes as detected by RT-PCR. RNA for IL-1, IL-4, IL-6, and GM-CSF were undetectable. The vast majority of the primary cultured stromal cells contained cytoplasmic IL-7 protein. By immunoperoxidase staining, mAb 8.28 revealed a reticular pattern in bone marrow sections. The stain was localized between hemopoietic cells in cluster-like formations scattered throughout the marrow. In a short-term culture assay for stromal cell frequency, 8.28 detected only a portion of identifiable stromal cells from fresh bone marrow suspensions. One possible explanation is that the lymphocyte-supporting stromal cells of Whitlock cultures represent a functional subset of hemopoietic reticular cells. To address this notion, we are assessing the co-localization in frozen marrow sections of $8.28^+$ cells and IL-7 protein, which has a reticular-like distribution similar to 8.28. (Supported by ACS grant IM-612. PLW is recipient of JRFA-409 from the American Cancer Society; PEF is a Loyola University Schmitt Fellow.)

*Surrogate Light Chains*

**FZ 246** THE FUNCTION AND EXPRESSION OF λ5 IN NORMAL MURINE B LYMPHOCYTE ONTOGENY, Alexis N. Bossie, Richard L. Riley, and Bonnie B. Blomberg, Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, FL 33101

The λ5 molecule is present on the surface of transformed pro- and pre-B cell lines, but is lacking from the surface of normal B lymphocytes. To determine its distribution in normal cells in the B lineage we examined RNA from normal pro- and pre-B cells that were expanded in culture with IL-7 and from normal pro- and pre-B cells freshly isolated from the bone marrow. Expression of λ5 mRNA in these cells was detected with the use of RT and PCR amplification. Freshly isolated, normal pro- and pre-B cells express roughly equivalent amounts of λ5 RNA and the level of expression remains constant through 10 days in culture with IL-7 as the cells differentiate from early to late pre-B cells. The level of λ5 RNA expression is approximately one to two orders of magnitude below the level of λ5 RNA expressed by the pre-B cell line 70Z. The λ5 protein cannot be detected on the surface of the freshly isolated or IL-7-cultured pro- and pre-B cells by conventional flow cytometry, but can be detected within the cytoplasm of these cells with the use of fluorescence microscopy. To investigate the function of the λ5 protein in early B lymphocyte lineage cells, pro- and pre-B cells were cultured in the presence of an anti-sense oligonucleotide to inhibit the translation of λ5 mRNA. 70Z cells constitutively express λ5 and μ on the cell surface and can progress to the expression of κ light chains. Culture of 70Z cells in the presence of the oligonucleotide resulted in an inhibition of cell surface expression of both λ5 and κ light chains. This method of inhibition of λ5 expression is currently being applied to the examination of the function of λ5 in normal and Abelson transformed pro- and pre-B cells and will provide a useful tool for the investigation of early B cell development in cells lacking the expression of this molecule.

**FZ 247** EXPRESSION OF AND SIGNAL TRANSDUCTION BY THE μ-SURROGATE LIGHT CHAIN COMPLEX ON HUMAN B CELL PRECURSORS, Helen C. Genevier, David J. Matthews and Robin E. Callard, Cellular Immunology Unit, Institute of Child Health, University of London, 30, Guilford Street, London WC1N 1EH, G.B.

Immunoglobulin μ chain is expressed on the surface of pre-B cells before conventional light chain production, in association with surrogate light chains. The μ-surrogate light chain complex appears to have an important role in B cell development. We have investigated expression of the surrogate immunoglobulin complex and signal transduction following antibody ligation in a panel of B cell precursor lines, in normal bone marrow, and in bone marrow from patients with X-linked agammaglobulinemia (XLA), an immunodeficiency in which B cell development is arrested at the pre-B cell stage.

Expression of surrogate light chain genes, VpreB and λ14.1, was demonstrated in most of the B cell precursor lines tested, using cDNA PCR and Northern analysis. In contrast, only 3 out of 9 lines expressed μ on the cell surface, in the absence of kappa and lambda light chains. Two of these 3 lines generated a $Ca^{2+}$ signal on ligation of surface μ by anti-μ antibody. Tyrosine phosphorylation was also induced by surface μ ligation. The capacity of this complex to transduce signals to the cytosol supports the hypothesis that its surface expression is important in B cell development.

VpreB and λ14.1 gene expression was demonstrated in the bone marrow of normal children and adults, and in XLA bone marrow. The role of the μ-surrogate light chain complex in XLA was further investigated by comparing its signal transduction properties to those in normal pre-B cells.

**FZ 248** THE ROLE OF "SURROGATE LIGHT CHAINS" IN B-CELLS, Joseph Haimovich, Efrat Rabinovich, and Mahmud Taya. Sackler School of Medicine, Tel-Aviv University. Tel-Aviv 69978, Israel.

Cells of B-lymphocyte cell lines, representing early stages of B-cell differentiation, synthesize two forms of IgM, a cell-surface membrane form and a secretary form. The former is quantitatively deposited on the cell surface whereas the majority of the latter is intracellularly degraded. Light-chain - defficient variants of early B-lymphocyte cell lines synthesize the "surrogate" light chains characteristic of pre-B cells. These surrogate light chains assemble with the μ heavy chains into IgM-like molecules which do not reach their destination and are intracellularly degraded. Interestingly, in light chain defficient variant cell lines "secondary rearrangements" occur resulting in the reexpression of membrane IgM, albeit with an idiotype different than that of the "original" IgM of the parental cell line. It is suggested that the surrogate light-chain - containing IgM-like molecules play a role in this secondary rearrangement. The same is probably true also in the "primary rearrangement" which results in the differentiation of pre-B into B-cells. It is also suggested that the process of secondary rearrangement plays a role in the expansion of the idiotype repertoire with a limited number of B-cell clones.

**FZ 249** SIGNALING THROUGH LAMBDA5 COMPLEXES ON PRE-B CELLS. J.Jongstra, F.Shinjo, V.Misener and W.-M.Zhu. Department of Immunology, University of Toronto and Toronto Western Hospital, Toronto, Canada, M5T 2S8.

Early mouse pre-B cell lines which do not express intact IgM-H protein (mu-protein), express a cell surface protein complex consisting of the pre-B cell specific proteins lambda5 and Vpre-B. We will present evidence that a 145 Kd protein (p145) is present as a third component of this complex. Late pre-B cell lines which express intact mu-protein, express a lambda5/Vpre-B/mu-protein complex on the surface. Early $mu^-$ pre-B cells showed a rapid and transient increase in intracellular free $Ca^{2+}$ when incubated with anti-lambda5 antibodies but not with anti-mu. Late $mu^+$ pre-B cell lines showed a similar increase in intracellular $Ca^{2+}$ after incubation with anti-lambda5 or anti-mu. Other responses of pre-B cells to engagement of the lambda5 complexes are currently investigated.

Thus, using a series of transformed pre-B cell lines as a model system we show the developmentally regulated appearance of different lambda5 complexes on the surface of pre-B cells and show that both complexes can transduce a $Ca^{2+}$-mobilizing signal to the inside of the cell. We are currently developing lambda5 specific monoclonal antibodies to investigate the surface expression of these complexes on normal pre-B cells from bone marrow or fetal liver.

FZ 250 BIOSYNTHESIS, CELL SURFACE EXPRESSION AND SIGNAL TRANSDUCTION OF THE μ-ΨL CHAIN COMPLEX IN HUMAN PRE-B AND INTERMEDIATE PRE-B-B CELLS. Claudine Schiff, David Bossy, Jean Salamero, and Michel Fougereau. Centre d'Immunologie Marseille-Luminy, France.

Pre-B cells can be characterized by the presence of the μ-ψL complex both in the cytoplasm and at the cell surface. Using λ-like and V pre-B specific antibodies, we have shown that association of the light chain surrogates to the μ chain occurs early after the biosynthesis in ER. Transport towards the cell surface has been followed by the analysis of μ chains maturation, as appreciated from glycosylation patterns. The V pre-B chain may exist in two forms and was found transiently associated with polypeptides of 17.5 and 36 kDa. λ–like-V pre-B complexes free of μ were also identified in the cytoplasm and were secreted. One cell line co-expressing μ–ψL and sIgM molecules has been identified and represents an intermediate pre-B/B stage of differentiation. Activation of pre-B and intermediate cells by an anti-μ yielded similar induction of $Ca^{++}$ flux and tyrosyl phosphorylation patterns, differing from those of the B cell suggesting that the intermediate cell stage lacks transducing cascade components necessary to achieve the B cell function. Finally attempts to isolate a soluble form of the μ–ψL complex were made by transfection experiments to try to identified a possible ligand on bone marrow stroma cells.

*Ig Gene Rearrangement Mechanisms*

**FZ 300 DETECTABILITY AND HETEROGENEITY OF IMMUNOGLOBULIN HEAVY CHAIN GENE REARRANGEMENTS IS DEPENDENT ON THE USED JOINING GENE PROBE**

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During B-cell differentiation rearrangements of immunoglobulin (Ig) variable (V), (diversity (D)), and joining (J) gene segments occur, resulting in specific V-(D-)J combinations, which are different in each B-cell. Since the various types of B-cell malignancies are regarded as clonal malignant counterparts of normal B-cells, analysis of Ig gene rearrangements can be used to establish clonality and differentiation state of B-cell malignancies.

We examined 80 precursor B-ALL for the configuration of their Ig genes at diagnosis and we performed comparative studies on Ig gene rearrangement patterns in 20 of these precursor B-ALL at diagnosis and subsequent relapse. At diagnosis multiple rearranged bands, often differing in density, were found in 38% of cases at the Ig heavy chain (IgH) gene level and in 6% of cases at the Ig kappa chain gene level. We could exclude hyperdiploidy of chromosome 14 in case of IgH genes and hyperdiploidy of chromosome 2 in case of Ig kappa genes as a cause of these multiple rearranged bands. Such high percentages of subclone formation (bi-/oligoclonality) have not been reported by other research groups. This could be due to the choice of restriction enzyme and/or probes, used for Ig gene analysis. Detailed studies on JH gene probes in various restriction enzyme digests revealed that the best results are obtained in *Bgl*II and *Bam*HI/*Hind*III digests using a JH probe which recognizes sequences just 3′ of the JH region (e.g. IGJH probe), whereas *Eco*RI and *Hind*III digests and the so-called JH-*Sau*3A probe (recognizing sequences of the JH2-6 region) give inferior results.

Differences in Ig gene rearrangement patterns between diagnosis and relapse were found in 55% of the 20 precursor B-ALL (including eight bi-/oligoclonal ALL). In one patient no oligoclonality at diagnosis and no clonal relationship between diagnosis and relapse could be found by use of the JH-*Sau*3A probe, whereas oligoclonality at diagnosis and clonal relationship between diagnosis and relapse was established by use of the IGJH probe.

Therefore, we conclude that optimal detection of clonality and heterogeneity in precursor B-ALL using IgH gene rearrangements is only possible by use of JH probes which are complementary to the 3′ side of the JH gene region. Most probably this also applies to J probes for analysis of other Ig and T-cell receptor genes.

*This work was supported by the Dutch Cancer Foundation (Nederlandse Kankerbestrijding, Koningin Wilhelmina Fonds), grant IKR 89-09*

FZ 301 ALTERNATIVE REARRANGEMENT OF LAMBDA AND KAPPA LIGHT CHAIN GENES IN NORMAL CIRCULATING AND TISSULAR MUCOSAL B CELLS. Marie C.BENE, Jianquing TANG, Claire MOLE and Gilbert C.FAURE. Laboratoire d'Immunologie, Faculté de Médecine, Université de Nancy I, BP 184, 54500 Vandoeuvre les Nancy, France.

The predominance of kappa versus lambda light chain, characteristic of serum immunoglobulins, is not found in mucosal immunoglobulins, either at the plasma cell level or in such secretions as milk, saliva or tears. According to rearrangements studies of light chain genes in malignant B cells, a hierarchy favouring this kappa light chain production is commonly admitted. However, other data from leukemias, lymphomas and cell lines indicate that lambda light chain genes could undergo rearrangements before kappa.

Purified B cells were obtained from 10 freshly removed human tonsils, after discarding T cells by E-rosetting. Kappa and lambda B cells were purified using antibody-coated magnetic beads. A non-isotopic Southern blot technique was applied to the DNA of both these B cells subsets and of controls (placenta and purified CD8+ cells) using specific kappa, lambda and CD4 probes. In control DNA, both kappa and lambda genes were found in germinal configuration. In purified tonsillar kappa-producing cells, the lambda probes displayed germline patterns. However, in lambda-producing cells, the kappa probe evidenced germline as well as rearranged bands, instead of rearrangement or deletion of both kappa alleles.

DNA from peripheral blood lambda B cells, similarly isolated, was submitted to a PCR technique, using primers specific for germinal kappa genes. A band of the prognosticated size could be amplified in lambda-producing cells as in kappa or in CD8 T cells, indicating again that at least a kappa gene might remain in germline configuration in those cells.

This indicates that lambda producing B cells test and might rearrange lambda genes before both kappa genes have been rearranged. Normal B cells, particularly from mucosal tissues, may thus follow light chain rearrangements patterns different from commonly admitted phenomena occuring in the systemic immune system. Results from peripheral blood B cells favour the hypothesis that many of them are travelling to and from mucosal areas.

FZ 302 IGH INTRONIC ENHANCER CONTROLS THE REARRANGEMENT, TRANSCRIPTION AND METHYLATION STATUS OF THE $J_H$ LOCUS, Jianzhu Chen, Fay Young, Andrea Bottaro, Valerie Stewart, & Frederick W. Alt, HHMI, Children's Hospital and Department of Genetics, Harvard Medical School, 320 Longwood Avenue, Boston, MA 02115

VDJ recombination in both precursor B and T lymphocytes is mediated by a common recombinase, however, the assembly is a tissue-specific process by which the complete assembly of immunoglobulin (Ig) variable region gene, for instance, occurs only in B cells not in T cells. Previous studies have shown that the IgH intronic Eμ enhancer can direct the tissue-specific rearrangement at a heterologous locus. In this study, we have replaced the 1 kb Xba I fragment containing the Eμ enhancer with an expressed *neo* gene on one allele through homologous recombination in embryonic stem (ES) cells. A-MuLV-transformed bone marrow pre-B cell lines were derived from chimeric mice that were generated from the injection of the targeted ES cells into blastocysts and the subsequent transfer of the blastocysts into foster mothers. Studies with over thirty ES cell-derived pre-B cell lines showed that the targeted allele virtually was not rearranged while the normal allele all had at least $D_H$ to $J_H$ rearrangement. Furthermore, the targeted allele was hypermethylated and absent of germline transcription. Thus, the Eμ enhancer is an essential cis-acting regulatory element for rearrangement, germline transcription and demethylation at the $J_H$ locus.

FZ 303 VDJ RECOMBINATION IN MOUSE THYMOCYTES: AGE-DEPENDENT INCREASE OF ABNORMAL DELETIONS IN REARRANGED TCRδ GENES, Suzanne M. Fish and Melvin J. Bosma, Institute for Cancer Research, Fox Chase Center Center, Philadelphia, PA 19111

Recombination products (RP) resulting from Dδ2 to Jδ1 (or Dβ1 to Jβ2) rearrangement in C.B-17 thymocytes were analyzed using PCR technology. The primers used allowed for deletions of ~100 nucleotides from the respective coding ends. Eleven of 15 RP (73%) from adult C.B-17 mice were found to contain abnormal deletions of Dδ2 and/or Jδ1 [as opposed to 0 of 10 RP resulting from Dβ1 to Jβ2 rearrangement]. An additional 5 RP contained N additions and/or P insertions between Dδ2 and its 5' signal sequence. These "open and shut" joints were abnormal, however, in that nucleotides from the signal sequence (3-9 bp) were deleted. A much lower frequency of abnormal Dδ2 and/or Jδ1 deletions was seen in RP from neonatal C.B-17 mice (1/10 RP). Similar results were obtained with C.B-17 scid mice; i.e., the frequency of abnormal Dδ2 and/or Jδ1 deletions was lower in RP recovered from neonatal mice (5/13 RP) than from adult mice (9/12 RP). Whether the abnormal RP in adult C.B-17 mice derive from TCRδ excision products (circular DNA) resulting from TCRα rearrangement, or reflect a marked increase in a particular T cell population, is unclear. However, neither of these possibilties would seem to apply in scid mice where T cell development is arrested at an early stage and TCRα genes are transciptionally inactive. We therefore suggest that the age-dependent increase in frequency of abnormal TCRδ rearrangements involving Dδ2 and Jδ1 may reflect a qualitative change in the recombinase activity. This could serve to down regulate the development of TCRγ/δ- expressing T cells.

FZ 304 THE RECOMBINATION ACTIVATING GENES OF THE RABBIT: EXPRESSION AND FUNCTIONAL STUDIES, Patrizia Fuschiotti, Moshe Sadofsky*, Joanne E. Hesse* and Rose G. Mage, LI, NIAID, and *LMB, NIDDK, NIH, Bethesda, MD 20892

The Recombination Activating Genes RAG-1 and RAG-2 of the mouse and human appear to be necessary components for the machinery needed for Ig and TCR gene rearrangements that occur in developing B and T lymphocytes. In earlier studies the rabbit RAG locus was cloned and the coding regions of the genomic RAG-1 and RAG-2 characterized. These studies showed that the organization of the rabbit RAG-1 and RAG-2 genes is similar to that reported for mouse, human and chicken and there is considerable conservation in sequence between species. We obtained evidence for allelic forms of rabbit RAG locus and, at least in some rabbits, there appears to be a duplicated copy of the RAG-2 gene. We isolated and sequenced two independent cDNA clones from thymic mRNA, each containing the complete coding region of the rabbit RAG-2 gene, by reverse transcription and PCR amplification. Sequence comparison between the two cDNAs and a genomic clone from another rabbit showed that both cDNA sequences differ from the genomic sequence at a few positions that likely reflect genetic differences between rabbits. The two cDNAs differ from each other in some positions that could also reflect different allelic forms of the rabbit RAG-2 gene. Southern analyses and PCR also suggested polymorphism of the RAG-2 locus. We analyzed the ability of the rabbit RAG-2 cDNA clones in combination with mouse RAG-1 to induce V(D)J recombination of an introduced recombination substrate fibroblasts. We found that two expression constructs with different sequences which may represent products of alleles or duplicated forms of RAG-2 were both able to induce recombination with an efficiency comparable to that of the mouse RAG-1 and RAG-2 gene control. Further exploration of functional activities of the RAG-2 genes in relation to the complex organization of the rabbit RAG-2 locus is in progress.

FZ 305 **REGULATION OF KAPPA LIGHT CHAIN GENE TRANSCRIPTION IN NORMAL PRE-B CELLS.** Sheila J. Gerety, Chris A. Klug, and Harinder Singh. Howard Hughes Medical Institute, University of Chicago, Chicago, IL 60637

We are analyzing the regulation of kappa gene transcription in normal pre-B cells using the Whitlock-Witte lymphoid bone marrow culture system and exogenous IL-7. By analysis of surface marker expression, this population is BP-1+, IgM-, and Thy1.2$^{lo}$, and the majority are B220+. Western blots reveal the expression of mu but not kappa protein. Rag-1 gene expression is on, further indicating that the cells are at the pre-B stage. In contrast with transformed pre-B cell lines, these normal pre-B cells show high level constitutive germline kappa gene transcription. This appears not to be directly due to the IL-7 added, since washout experiments did not diminish the level of kappa transcription. The majority of NF-κB in these cells is in the activated form, again in contrast to the level of active NF-κB normally found in unstimulated transformed pre-B cell lines. A panel of kappa promoter- and enhancer-containing reporter gene constructs are being introduced into these nontransformed pre-B cells. This will allow examination of the functional importance of previously identified regulatory elements in controlling kappa gene transcription in normal, pre-B stage cells.

**FZ 306** V(D)J CODING JOINT FORMATION: SEQUENCE AT CODING ENDS CAN MARKEDLY AFFECT THE OUTCOME OF V(D)J RECOMBINATION.

Rachel M. Gerstein and Michael R. Lieber. Department of Pathology, Stanford University Medical Center, Stanford, CA 94305-5324.

The variable region exon of antigen receptor genes is created by V(D)J recombination. The process of coding end joining, with its attendant nucleotide loss and addition, is a critical aspect of the generation of a diverse repertoire. Considerable attention has been given recently to the possibility that junctional diversity may be limited by the usage of short regions of homology in the joining of coding ends. Using a system that is free from the several levels of cellular selection (extrachromosomal V(D)J recombination substrates), we find that the diversity of coding joints can be severely restricted when the coding ends participating in the reaction have a short region of homology. This mechanistic feature has significant implications for immune repertoire development.

Though base-pairing between the two coding ends can constrain the diversity of coding joints, we have found that it is not required. Additional substrates were tested that demonstrate that coding end sequence can have a marked negative impact on recombination. We conclude that coding end sequences can affect the extent of diversity generated as well as quantitative aspects of coding joint formation.

**FZ 307** ROLE OF $C_pG$ METHYLATION IN CONTROL OF IG KAPPA GENE REARRANGEMENT, Michele Goodhardt, Patricia Cavelier, Noëlle Doyen, Charles Babinet and François Rougeon, Département d'Immunologie, Institut Pasteur, Paris, France

We have previously shown that unlike endogenous kappa genes, exogenous unrearranged kappa transgenes undergo $V_k$-$J_k$ recombination in T cells of transgenic mice. In order to determine whether the difference in B/T cell specificity in the rearrangement of the transgenic and endogenous kappa genes is underlied by differences in chromatin structure, the methylation status of the endogenous and transgenic genes was compared in the thymus of transgenic animals. For the transgenic kappa genes, the $J_k$-$C_k$ region was totally or partially cut by the methylation-sensitive enzymes HhaI and HpaII. In contrast, the endogenous $J_k$-$C_k$ locus is totally resistant to HhaI cleavage at the same sites. These results indicate that there is an inverse relationship between DNA methylation and $V_k$-$J_k$ recombination. This idea is reinforced by the observations that the endogenous $J_k$-$C_k$ locus is hypomethylated in B cells and preB cells undergoing kappa gene rearrangement. In contrast, the kappa locus is fully methylated in preT and T cells, as well as B cell precursers undergoing Ig heavy, but not light chain gene rearrangement. To test the hypothesis that hypomethylated kappa genes are constituatively accessible to recombination *trans*-acting factors, fibroblasts from transgenic mice were transfected with the recombination activating genes Rag1 and Rag2. Following transfection, recombination of the hypomethylated, but not the methylated kappa genes was observed.

Taken together, these results suggest that hypomethylation correlates with a more accessible state of the kappa locus and that methylation / demethylation may be important in the control of kappa gene rearrangement during lymphocyte differentiation.

**FZ 308** IDENTIFICATION OF AN *IN VITRO* V(D)J RECOMBINATIONAL SIGNAL SEQUENCE DEPENDENT DNA JOINING ACTIVITY FROM LYMPHOID NUCLEAR EXTRACTS.

Thomas G. Guilliams, Ming Teng, J. Brian Nauert, Nadine L. N. Halligan, and Brian D. Halligan. Department of Microbiology, Medical College of Wisconsin, Milwaukee WI 53226

Immunoglobulin gene segment assembly occurs through a site-specific recombination process known as V(D)J recombination. Using a new *in vitro* RSS dependent DNA joining assay, a DNA joining activity has been identified from extracts of lymphoid cell nuclei. This activity is V(D)J signal sequence dependent and similar to that of a bacterially expressed recombinant protein called VDJP (V(D)J DNA Joining Protein). This activity is lymphoid specific and has not been seen in non-lymphoid cell lines. Sequences of products from these reactions are being further analyzed for joining precision. Using sequences from VDJP, experiments in which the abundance of this nuclear protein is reduced by antisense oligonucleotides or germline gene insertion are being performed to test the role of VDJP in the recombination of extrachromosomal DNA. VDJP constructs are also being introduced into non-lymphoid cells such as fibroblasts. These cells will be tested for their ability to recombine extrachromosomal DNA and nuclear extracts will be assayed for V(D)J DNA joining activity. The pattern of expression of VDJP and the structure of the gene for VDJP are also under investigation. These studies will lead to an understanding of the role of VDJP in lymphoid V(D)J recombination.

**FZ 309** HUMAN COMMON ACUTE LYMPHOBLASTIC LEUKEMIA DERIVED CELL LINES ARE COMPETENT TO RECOMBINE THEIR T CELL RECEPTOR $\delta/\alpha$ REGIONS ALONG A HIERARCHICALLY ORDERED PATHWAY

Thomas E. Hansen-Hagge, Shouhei Yokota, Hubertus J. Reuter, Klaus Schwarz, and Claus R. Bartram, Section of Molecular Biology, Department of Pediatrics II, University of Ulm, FRG.

Normal as well as malignant pre-B and pre-T lymphocytes seem to possess a capacity to execute incomplete cross-lineage recombinations at their TCR and Ig loci, respectively. The majority of all common acute lymphoblastic leukemias (cALL; pre-B leukemia) displays a Vδ2/Dδ3 rearrangement at the TCR δ locus, which is also found in normal blood cells at a remarkably high frequency (0.01 to 0.1%). Moreover, 20 % of cALL cases have joined the Vδ2Dδ3 segment to one of the Jα-elements, and consequently deleted most of the TCR δ locus, which is nested within the TCR α locus. We have speculated that Vδ2Dδ3Jα hybrid molecules represent potential intermediates along a VαJα recombination pathway normally present in pre-T cells. In fact, there are striking similarities between this hypothetical pathway and the δRec/ψJα recombination model, with δRec and ψJα as nonfunctional elements flanking the TCR δ locus. The assembly of δRec and ψJα initiates TCR α rearrangement by subsequent replacement of the intermediate by functional VαJα recombinations. We have identified two cALL derived cell lines, REH and Nalm-6, which continuously recombine preexisting TCR δ fusion products to Jα gene segments under conventional tissue culture conditions. Analysis of different REH subclones obtained by limiting dilution of the initial culture, revealed a biased recombination of Vδ2Dδ3 to distinct Jα elements. During prolonged tissue culture a subclone acquired growth advantage and displaced parental cells as well as other subclones. Frequently, the DJ junctions of REH subclones contained extended stretches of palindromic sequences derived from modified Dδ3 coding elements. The other cell line, Nalm-6, started the TCR δ/α recombination with an unusual signal joint of a cryptic recombinase signal sequence (RSS) upstream of Dδ3 to the 3' RSS of Dδ3. The RSS dimer was subsequently rearranged in all investigated subclones to an identical Jα element.

FZ 310 ANALYSIS OF THE DEFECT IN DNA END JOINING IN THE MURINE SCID MUTATION, John Harrington, Chih-Lin Hsieh, Jennifer Gerton, Gayle Bosma, and Michael Lieber, Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305-5324

Murine severe combined immune deficiency (scid) is characterized by a 5000 fold reduction in coding joint formation in V(D)J recombination and a sensitivity to DNA damaging agents such as ionizing radiation and bleomycin. Furthermore, we have observed that the stable integration of linear DNA into scid fibroblasts is reduced 11 to 75 fold compared to normal fibroblasts. In order to investigate the link between V(D)J coding joint formation and general end joining, we carried out a series of experiments in scid and normal cells which were transfected with linearized plasmids. We find that intermolecular and intramolecular end joining are both quantitatively and qualitatively normal in scid lymphocytes and fibroblasts. We discuss the types of end joining reactions that are and are not affected in this defect in the context of a hairpin model for V(D)J recombination. Currently, we are developing biochemical assays for V(D)J recombination which will allow us to test this model.

FZ 311 HAIRPIN INTERMEDIATES IN V(D)J JOINING Susanna M. Lewis, Joseph T. Meier, and Linda L. Czyzyk. Division of Biology, California Institute of Technology, Pasadena, CA 91125

A subset of V(D)J junctions, comprised of untrimmed "coding ends" contain junctional inserts, termed "P" (for palindromic) nucleotides. These inserted residues create short (usually 1-4) stretches of sequence with an inverse complementary relationship to the adjacent coding end terminus. P inserts have been proposed to arise as a consequence of an obligate hairpin formation step in V(D)J joining.

We have investigated the generality of the P insert phenomenon in an experimentally manipulable system, and can demonstrate by stringent statistical tests that P insertion is a characteristic, if irregular, feature of the V(D)J joining process. This irregularity is best explained by the hairpin model.

We have further tested the possibility that P inserts are derived from hairpin ends by directly introducing hairpin-terminated DNA into cells. Resolved hairpin ends are recovered as recircularized molecules which are then subjected to DNA sequence analysis. Our data support a hairpin origin for P inserts.

Hairpin resolution was compared in cells derived from normal and *scid* mice in order to determine whether or not the *scid* lesion might cause a hairpin-processing defect. Preliminary evidence suggests that the *scid* function is not specifically involved in opening covalently-closed DNA termini.

FZ 312 ACTIVITY AND STABILITY OF RAG-2 PROTEIN ARE REGULATED BY DISTINCT PHOSPHORYLATION PATHWAYS, Weei-Chin Lin and Stephen Desiderio, Department of Molecular Biology and Genetics and Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

V(D)J recombination can be activated in fibroblastoid cells by coexpression of the Recombination Activator Genes *RAG-1* and *RAG-2*. With the aid of specific anti-peptide antibodies, we have begun to study the proteins encoded by these genes. RAG-1 and RAG-2 proteins are phosphorylated on serine and threonine, and both are recovered primarily in the nuclear subcellular fraction. We have found that the stability and activity of RAG-2 are regulated by distinct phosphorylation pathways. Casein kinase II (CKII) phosphorylates bacterially expressed RAG-2 at multiple sites within the acidic region. Two of these sites correspond to the major sites of RAG-2 phosphorylation in vivo. An Ala substitution at one of these sites reduced the ability of RAG-2 to activate recombination of an extrachromosomal substrate in vivo, but did not alter the level of RAG-2 protein or impair its nuclear localization. Ala substitutions at five other Ser or Thr residues within the acidic domain had no effect on rearrangement. These observations suggest that the activity of RAG-2 is positively regulated by phosphorylation of a specific Ser residue within the acidic domain. The RAG-2 sequence reveals two potential $p34^{cdc2}$ phosphorylation sites; both of these are phosphorylated by $p34^{cdc2}$ or a related kinase in vitro. Substitution of Ala for Thr at one of these sites resulted in a remarkable increase in the steady state level of RAG-2 protein. The relative increase in RAG-2 expression is most likely the result of protein stabilization, because mutant and wild type RNA levels were identical and chimeric proteins containing wild type or mutant RAG-2 polypeptide segments exhibited a similar difference in steady state level. These results imply that phosphorylation of a specific Thr residue by $p34^{cdc2}$ or a related kinase targets RAG-2 for degradation. Consistent with this interpretation, in vivo phosphorylation of this Thr residue was not detectable in wild type RAG-2, but was readily detected in RAG-2 carrying a deletion that impairs $p34^{cdc2}$-dependent degradation. Our observations suggest that antigen receptor gene rearrangement is coupled to the cell cycle.

FZ 313 FUNCTIONAL ANALYSIS OF BACULOVIRUS-EXPRESSED RAG 1 AND RAG 2 PROTEINS, Bethany B. Moore and Katheryn Meek, Department of Internal Medicine, U.T. Southwestern, Dallas, TX 75235.

Three years ago, two highly conserved "recombinase activating genes" (RAG 1 and RAG 2) were identified by Shatz, Oettinger, and Baltimore. It has been surmised that the protein products of these genes are involved in V(D)J recombination. The transfection of these cDNAs can induce recombination of extrachromosomal substrates when expressed in fibroblasts; however, it is not known what other eukaryotic factors may be activated. To address whether or not RAG 1 and RAG 2 are sufficient to induce recombinase activity, we have expressed the RAG 1 and 2 cDNAs in a baculovirus system and assayed for the ability of these expressed proteins to mediate recombination of a baculovirus-based substrate in SF9 insect cells. The results were negative, suggesting that the RAG 1 and 2 proteins cannot mediate the event alone. However, there is one major caveat to this conclusion. We do not know for sure that the baculovirus expressed proteins are functional. In order to test this, we plan on assaying the baculovirus-expressed RAG 1 and 2 proteins in fibroblasts. To facilitate these experiments, we have optimized the conditions for general protein electroporation into fibroblasts using a quantitative β-galactosidase assay. Furthermore, we have optimized the conditions for specifically electroporating RAG 2 containing crude lysates. Western analysis has demonstrated that we are effectively introducing RAG 2 protein into the cells and that RAG 2 has an apparent half-life of 24 hours. We are currently working to optimize the conditions for electroporation of RAG 1 lysates. Experiments are currently underway to analyze the electroporated RAG 2 protein in a fibroblast line stably transfected with RAG 1 cDNA and substrate. Future studies include analysis of co-electroporated RAG 1 and 2 proteins.

FZ 314 CHARACTERIZATION OF STERILE IMMUNOGLOBULIN HEAVY CHAIN TRANSCRIPTS IN HUMAN LEUKEMIC MYELOID CELLS, Lynn C. Moscinski, Laura Williams and Peter G. Medveczky, Departments of Pathology and Medical Microbiology and Immunology, University of South Florida College of Medicine, Tampa FL 33612

Transcription of the immunoglobulin (Ig) locus is thought to precede gene rearrangement, and may be the initiating event. This process is regulated by trans- and cis-acting elements. The octamer ATTTGCAT, found in most Ig gene promoters and the mu heavy chain ($C\mu$) enhancer, represents one determinant of B cell specificity. Transcription at this locus initiates from the promoter upstream of the rearranged variable region. However, in addition to productive transcripts from the rearranged heavy chain gene, sterile $C\mu$ transcripts commonly originate from joining of the D-J segments or from the J-$C\mu$ intron. We have identified a spectrum of short $C\mu$ sterile transcripts, varying in size from 1.4-1.8 kb, in 83% of a series of 42 acute leukemias of myeloid origin. The presence of $C\mu$ sterile transcripts does not correlate with expression of either TdT or RAG-1, although mRNAs for both of these proteins are frequently identified. In an attempt to further characterize these transcripts, we have studied several in greater detail. We used a 300 bp IgH enhancer region cDNA, containing the octamer sequence, to probe Northern blots. A distinct hybridizing band could only be identified in the control pre-B cell line, despite the presence of $C\mu$, suggesting that the sterile transcripts found in myeloid cells do not contain this region. PCR analysis demonstrates normal splicing of the myeloid transcripts, as well as an intact 3' end containing a portion of the $C\mu$-membrane sequence. Primer extension suggests extreme heterogeneity in the 5' end, with multiple sites of transcription initiation. A second splice site is detected within the enhancer sequence. Our results indicate that the Ig gene locus is undergoing active transcription in neoplastic myeloid cells. This process produces a series of sterile $C\mu$ mRNAs which share several features in common with those of B cells. However, important differences in initiation site and possibly secondary splicing are noted.

FZ 315 ENDOGENOUS IMMUNOGLOBULIN GENE EXPRESSION DURING FETAL DEVELOPMENT OF IgA TRANSGENIC MICE. C. O'Connell[1], D. Longo[2], S. Hill[2], J. Hurst[1] and J. Kenny[1]. [1]PRI/DynCorp, Inc., Biological Carcinogenesis and Development Program, [2]Biological Response Modifiers Program, NCI-FCRDC, Frederick, MD 21702.

Allelic exclusion of endogenous immunoglobulin (Ig) gene expression occurs in transgenic (TG) mice containing $\mu$, $\delta$, and $\alpha$ transgenes. However, this feedback inhibition is generally incomplete, with up to 20% of the B cells in $TG^+$ animals co-expressing endogenous and transgene-encoded Ig. We sought to determine whether this incomplete exclusion of endogenous IgM was due to the timing of endogenous vs. transgenic Ig gene expression during B cell development. Since the order of heavy (H) and light (L) chain expression in fetal development duplicates the ordered expression in an individual B cell, we examined by reverse transcriptase PCR the kinetics of $\mu$, $\alpha$, and $\kappa$ expression in day 11-17 fetal tissue from IgA transgenic animals. This analysis revealed that the kinetics and apparent level of $\mu$ mRNA expression was unaltered in the IgA transgenic mice compared to $TG^-$ controls with $\mu$ mRNA detectable on day 12 in both fetuses. The $\alpha$ transgene was expressed on day 11. The failure of $\alpha$ expression to suppress $\mu$ mRNA synthesis could be due to: 1) a requirement of the B cell to pass through stages of development requiring $\mu$:pseudo-light chain signaling; or 2) a lack of $\alpha$ chain translation and surface expression prior to endogenous $\mu$ transcription. Mu:pseudo-L-chain expression is not mandatory since $\lambda 5$ mRNA was never detected in the transgenic mice but was easily detectable in the controls. The transgene encoded L-chain was detectable on day 12 while L-chain mRNA was first seen on day 16 in control fetuses. The kinetics of synthesis and surface expression of transgene encoded proteins during fetal development is under investigation.

FZ 316 GENETIC ANALYSIS OF RAG-1 AND RAG-2 Marjorie A. Oettinger, Susan Kirch and Christina Cuomo. Department of Molecular Biology, Massachusetts General Hospital and Department of Genetics, Harvard Medical School, Boston, MA 02114.

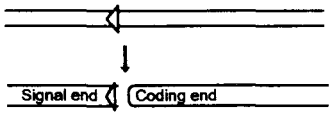
The recombination activating genes RAG-1 and RAG-2 are sufficient to induce V(D)J recombination in non-lymphoid cells and their expression is required for assembly of Igs and TCRs during lymphoid development. The actual role of the products of these genes in V(D)J recombination is not understood.

In an effort to learn more about their function, we have been carrying out mutational analyses of both RAG-1 and RAG-2 . The results of this analysis will be presented. Further, we have been using genetic techniques to identify genes whose products functionally interact with RAG-1 or RAG-2. The initial characterization of these clones will be discussed.

FZ 317 V(D)J RECOMBINATION SUBSTRATE TARGETING IN A RECOMBINASE-INDUCIBLE CELL SYSTEM, Oltz, E.M. Rathbun, G. Chen, J and Alt, F., Dept. of Gentics, Harvard Univ., Boston, MA 02115 Somatic recombination of V(D)J gene segments that encode the variable regions of immunoglobulin (Ig) and T-cell receptor (TCR) proteins is a highly regulated and ordered process. Since all rearrangement events are mediated by a common recombinase activity, the specificity of this process must be endowed by modulating accessibility of the substrate gene segments. Several lines of evidence have suggested a role for transcription in rendering accessibility, including studies with transgenic TCR-beta minilocus substrates which have conclusively demonstrated the ability of DNA segments that contain transcriptional control elements (i.e. the Ig heavy chain enhancer - $E_{\mu}$) to dominantly target rearrangements. In vitro systems to study the specifics of this targeting mechanism have been lacking, since transformed pre-lymphocyte cell lines express low and variable levels of recombinase activity. Therefore, we have constructed a novel cell system (DR37) that enables transient high level expression of the recombinase activating genes (RAG-1 and RAG-2) in the null background of a mature B cell. As in mice, rearrangement of TCR-B minilocus substrates proceeds in an efficient and regulated manner in the DR37 cell line (Eu+ rearranges and Eu- does not). We have further applied these methodologies to show that a minimal version of the Eu element, as well as the lymphoid-specific kappa intronic enhancer and the pan-specific SV40 enhancer are all capable of dominantly targeting substrates. Results will be presented from these and other substrates which replace enhancer elements with versions that are mutated at transcriptionally important motifs, or with an inducible promoter. These results will be discussed in the context of the role of transcription in targeting V(D)J rearrangement.

FZ 318 **V(D)J RECOMBINATION: BROKEN DNA WITH HAIRPIN CODING ENDS IN *scid* MOUSE THYMUS,**
D.B. Roth[1], J.P. Menetski[1], P.B. Nakajima[2], M.J. Bosma[2], and M. Gellert[1], 1. Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD 20892; 2. Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111.

V(D)J recombination events in the murine T cell receptor δ locus frequently involve the D2 and J1 elements. We have reported previously the presence of double strand breaks at recombination signals flanking D2 and J1 in 2% of thymus DNA from BALB/c mice (Roth et al., **Cell 69**: 41-53, 1992). Only broken DNA with signal ends was detected; no species with coding ends could be identified. Because the murine severe combined immunodeficiency (*scid)* mutation is thought to impair specifically the joining of coding ends, we searched for molecules with coding ends in thymocyte DNA from scid mice. In this DNA, broken molecules with coding ends (as well as molecules with signal ends) can indeed be found. Remarkably, these coding (but not signal) ends are covalently sealed into hairpin structures.




The formation of hairpins at coding ends may be a universal, early step in V(D)J recombination. This would provide a simple explanation for the frequent short palindromic (P) nucleotide insertions at coding joints from wild-type cells, and the rare, but much longer, P nucleotide inserts at coding joints from scid cells. The *scid* factor may be required for the proper cleavage of hairpins; rare, random nicks may substitute for its activity.

FZ 319 EXPRESSION OF RAG-1 AND RAG-2 mRNA IN RABBIT LYMPHOID TISSUE, Kenneth H. Roux, Gus Ray, and Wayne T. McCormack*, Department of Biological Science, Florida State University, Tallahassee, FL 32306, and *Center for Mammalian Genetics, University of Florida, College of Medicine, Gainesville, FL 32610.

The recombination activating genes,RAG-1 and RAG-2,have been implicated in the DNA splicing events occurring during VDJ rearrangement. In addition, RAG-2 expression in the absence of RAG-1 expression has been correlated with gene conversion of V region genes in the chicken. Because somatic diversity of the rearranged rabbit $V_H1$ gene is also generated by gene conversion, we used Northern and slot blot analysis to compare the level of RAG-1 and RAG-2 mRNA in lymphoid tissue of rabbits of various ages (newborn, 2 week, 6 week, 1 year). As anticipated, the thymus from rabbits at birth through adulthood were strongly positive for RAG-1 and RAG-2. In contrast, the spleen, lymph nodes, Peyer's patches, sacculus rotundus, and appendix were RAG-$1^-$RAG-$2^-$. The bone marrow from newborn, 2 week and 6 week old rabbits were moderately positive for RAG-2 displaying 10%, 7%, and 5% as much RAG-2 as the thymus, respectively. Interestingly, the RAG-1 signal was much weaker on a proportional basis or, in most cases, was not detectable in the bone marrow at these early ages. Adult bone marrow tested RAG-$1^-$RAG-$2^-$. These data are consistent with the notion that the RAG-$1^-$RAG-$2^+$ phenotype may be associated with gene conversion, and suggests that this activity may reside primarily in the bone marrow in young rabbits.

FZ 320 STATUS OF IG GENES IN PERITONEAL B CELL HYBRIDOMAS FROM LEAKY SCID MICE. Ruetsch,N.R., Debra Kotloff and Melvin Bosma, Fox Chase Cancer Center, Philadelphia, PA 19111

Twenty-one IgM(κ)-producing B cell hybridomas from the peritoneal cavity of old leaky Balb/c *scid* (scid) mice and 14 from age-matched control Balb/c mice were cloned and compared as to the status of their IgH and IgL(κ) loci. Strikingly, in the scid clones with two accountable IgH loci, the non-expressed alleles were either abnormally rearranged (5/14) or retained in germline configuration (9/14 vs 1/10 in the controls). The expressed IgH alleles in the 21 scid clones showed relatively high utilization of Vh genes (7183/Q52) proximal to the Dh locus (7/21 vs 2/14 in the controls) and over-representation of Jh4 (12/21 vs 4/14 in the controls). Recombination between regions of Vh/Dh or Dh/Jh homology (3-8 bp) occurred in 6 of the 21 alleles. Most of the non-expressed IgL(κ) loci in scid clones with two accountable alleles were unrearranged. Of the 21 expressed IgL(κ) alleles, 11 contained a Vκ4/5 gene and all but one contained Jκ1 or Jκ2. P nucleotide insertions were seen in 14/21 VJκ junctions (vs 3/12 in the controls). In 9 scid clones, the Vκ-Jκ recombination was apparently mediated by P nucleotide insertions derived from Jκ1 that were homologous to 4-5 bp in the germline sequence of Vκ4/5 members. These results suggest that the development of scid peritoneal B cells is favored by a minimal number of attempted rearrangements and by the use of certain heavy and light chain V, (D) and J elements, some of which may promote homologous recombination.

FZ 321 VDJ RECOMBINATION IN B CELLS IS IMPAIRED BUT NOT BLOCKED BY TARGETED DELETION OF THE IGH INTRON ENHANCER, Fred Sablitzky and Matthias Serwe, Max-Delbrück-Laboratorium in der MPG, Köln, Germany

In the course of B lymphocyte development the assembly of immunoglobulin (Ig) variable gene segments (V, D and J) in pro- and pre-B cells is a prerequisite for the expression of functional immunoglobulins. Onset, timing and specificity of the recombination process have to be strictly controlled. It is assumed that the level of accessibility of the Ig loci to the recombinase machinery is a key factor of this regulation. This model predicts the existence of control elements within the Ig loci which modulate the accessibility of nearby gene segments. Recent data suggested that such cis-regulatory elements are associated with sequences like enhancers, silencers and promoters that control the transcription of rearranged immunoglobulin genes. We have assessed the importance of the immunoglobulin heavy chain (IgH) intron enhancer for the recombination of variable gene segments during B cell development by generating chimeric mice with embryonic stem cells lacking the intron enhancer from one of the IgH loci. The IgH intron enhancer was substituted by a short oligonucleotide by homologous recombination using the Hit & Run procedure. Rearrangement of the variable gene segments of the mutant locus lacking the IgH intron enhancer was impaired but not blocked. Quantitative PCR analyses showed that 50% of the mutated IgH loci stayed in germline configuration, about 40% underwent D-to-J and 10% V-to-DJ recombination. The present results indicate that the accessibility of the immunoglobulin locus for the recombination machinery is influenced by, but not dependent upon the presence of the intron enhancer.

**FZ 322** N REGION ADDITION AND UNUSUAL CDR 3 LENGTH DISTRIBUTIONS IN κ-CHAIN ONLY PRE-B CELLS.

Harry W. Schroeder, Jr., Peter D. Burrows, Max D. Cooper, and Hiromi Kubagawa. Departments of Medicine, Microbiology, and Pathology, University of Alabama at Birmingham, Birmingham, AL 35294-3300.

Solution of the crystal structure of immunoglobulins has documented that the third complementarity determining regions (CDR) of the heavy and light chains create the center of the antigen binding site. Unlike the heavy chain, Vκ-Jκ junctions in the mouse are characterized by an absence of N region addition and a constant length (9 residues) of CDR 3. The light chain is thus the more limited partner in the production of a diverse antibody repertoire. N region addition is associated with terminal deoxynucleotidyl transferase (TdT) activity. TdT is tightly regulated during B cell development, usually being present in B lineage cells only prior to the production of functional heavy chain. Having shown previously that light chain gene rearrangement can precede heavy chain gene rearrangement in man, we postulated that these light chain rearrangements may occur in the presence of TdT and thus result in the introduction of non-templated nucleotides at the V-J junction. We cloned the genomic in-frame and out-of-frame Vκ-Jκ rearrangements from four κ-chain only EBV-transformed pre-B cells that had failed to rearrange VH. Of the seven rearrangements examined, four utilized Vκl family gene segments and three contained VκII elements. Among these seven VJ joins, six had N region addition and none of the rearrangements contained a 9 amino acid CDR 3. The introduction of N region addition into the VJ junction increases the potential diversity of the preimmune repertoire and has the potential to create unusual antigen binding sites. Although most human κ light chains lack N region addition and contain normal sized CDR 3 intervals, κ light chain transcripts from rheumatoid synovium and κ components of antibodies with rheumatoid factor activity often contain contain N region addition and a wide spectrum of CDR 3 lengths. Since B cells which rearrange light chain genes before heavy chain genes may have increased potential for the generation of high affinity self-reactive antibodies, we speculate that this population may contribute to the pathogenesis of diseases of immune function.

**FZ 323** **RECOMBINATION DEFECT IN B-CELL NEGATIVE (B⁻) SCID PATIENTS**, Klaus Schwarz, Thomas E. Hansen-Hagge and Claus R. Bartram, Section of Molecular Biology, Pediatrics II, University of Ulm, Prittwitzstraße 43, 7900 Ulm, Germany.

Human severe combined immunodeficiency is a term to describe a heterogeneous group of X-linked and autosomal genetic disorders characterized by lethal defects in both cellular and humoral immunity. Most SCID patients exhibit detectable, albeit functionally ineffective B cells. About 30% of affected infants have defects in the purine salvage pathway, in the expression of histocompatibility genes or in the interleukine production and response. However, the majority of SCID patients still awaits further characterization at the molecular level.

This is in contrast to the classical scid mouse model, where a a singele mutation results in defective Ig and T cell receptor rearrangements. Moreover, transformed B-precursor cell lines are not available for molecular analyses from SCID patients characterized by the complete absence of B and pre-B cells (B⁻ SCID). To study the V(D)J recombinase machinery in this SCID entity we have developed a PCR assay which enables us to characterize simultaneously all six possible DHQ52-JH rearrangements of the Ig heavy chain locus. B⁺ SCIDS exhibited a normal recombination pattern. In contrast, B⁻ SCID patients showed a grossly altered DHQ52-JH rearrangement pattern characterized by the total or partial absence of regular recombinations and the presence of abnormal rearrangements. Sequence analyses demonstrated that the abnormal events were caused by deletions surpassing the boundaries of Ig coding elements. Thus, according to theses analyses, B⁻ SCID patients resemble molecularly either RAG 1&2 deficient "knock out" mice (absence of recombination), or the classical scid mouse (faulty recombination).

**FZ 324** THE V(D)J RECOMBINATION-ACTIVATING GENES RAG-1 AND RAG-2: DISTRIBUTION, PHENOTYPE OF RAG-1 DEFICIENT MICE AND THE REGULATORY ROLE OF ANTIGEN RECEPTORS. Eugenia Spanopoulou, Christopher Roman, Patricia Cortes, Dan Silver, Michele Nussenzweig, Dimitris Kioussis", Richard Hardy*, Lynn Corcoran# and David Baltimore. The Rockefeller University, New York, NY 10021, " NIMR, Mill Hill, London NW7, UK., * Fox Chase Cancer Center, Philadelphia, PA 19111, # WEHI, Victoria 3050 Australia.

Variable regions of diverse antigen-binding specificity are created when different V, (D) and J coding elements are joined by somatic recombination. Two genes, RAG-1 and RAG-2, were identified in our laboratory, the products of which can promote V(D)J rearrangement on a recombination substrate. To further characterize their distribution pattern, we developed antibodies against the *RAG-1* and *RAG-2* proteins and used them in Western and immunoprecipitation assays. These studies revealed that both proteins are phosphorylated and present at very low levels in lysates from cell lines competent in V(D)J recombination. *RAG-1* localizes as speckles in the nucleus, whereas *RAG-2* exhibits a very even nuclear distribution pattern.

In an attempt to follow the function of the *RAG-1* protein, the *RAG-1* allele was interrupted by homologous recombination. Mice homozygous for the mutation are immunodeficient since they fail to produce any functional B- or T-lymphocytes. Both types of cells are arrested at any early stage before the rearrangement of immunoglobulin or T-cell receptor molecules. No V(D)J recombination events were detected in these mice indicating that unlike the *scid/scid* phenotype the absence of *RAG-1* does not permit any functional rearrangements to occur.

The availability of "non-leaky" immunodeficient mice provides a system to study the regulatory role of antigen receptors in B- and T-cell development. Thus, the *RAG-1* deficient mice were backcrossed to mice transgenic for a rearranged IgH coding unit. The mice are expected to present a single receptor on the surface of B-cells and the ability of this receptor to support B-cell differentiation will be studied. Similarly, we have generated *RAG-1* deficient mice carrying a rearranged TcRαβ receptor of known specificity in order to study the regulatory role of the receptor in T-cell development. The results of these studies will be described.

**FZ 325** UPREGULATED RAG GENE EXPRESSION IN SURFACE IMMUNOGLOBULIN NEGATIVE VARIANTS OF A HUMAN B CELL LINE, Niclas B.J. Stiernholm and Neil L. Berinstein, Department of Immunology, University of Toronto, Toronto, ON M4Y 1L8, CANADA.

In order to study the potential association between sIg expression and the expression of the RAG genes, we isolated sIg⁻(λ⁻) variants of a human mature sIg⁺ B cell line. Northern analysis revealed that while the parental cell line had low RAG expression, the sIg⁻ variants had significantly upregulated their expression of the RAG1 and RAG2 genes. It was further discovered that the entire recombination machineries of these variants were activated and that they consequently were capable of carrying out secondary Igλ rearrangements and thereby regenerate sIg expression. The resulting sIg⁺ progenies differed from the parental cell line both in their idiotypes and their Cλ isotypes. In spite of regained sIg expression, the elevated levels of RAG expression seen in the sIg⁻ variants, remained in the sIg⁺ variants. However, further Igλ rearrangement activity could not be detected. These results indicate that while the induction of RAG expression in the sIg⁻ variants may be inversely associated with sIg expression, sIg expression alone, is not sufficient to downregulate RAG expression in the sIg⁺ variants. This cell culture system provides a system which can be experimentally manipulated to further dissect the components involved in the regulation of Ig gene rearrangement activity.

FZ 326 FUNCTIONAL ANALYSIS OF A cDNA ENCODED V(D)J DNA JOINING PROTEIN (VDJP) WITH RECOMBINATIONAL SIGNAL SEQUENCE (RSS) SPECIFIC DNA JOINING ACTIVITY

Ming Teng, Tom Guilliams, J. Brian Nauert, Nadine L. N. Halligan, and Brian D. Halligan, Department of Microbiology, Medical College of Wisconsin, Milwaukee, WI 53226

Immunoglobulin (Ig) genes are assembled by the site-specific conservative V(D)J recombination process which is mediated by recombinational signal sequences (RSS). A cDNA encoding VDJP is a DNA binding protein that specifically binds to the nonamer sequence of the RSS. VDJP has been cloned and recombinant VDJP has been expressed in *E. coli* and tested for RSS dependent DNA joining activity. Predicted amino acid sequence at the carboxyl terminus of VDJP was found to be highly homologous to the amino acid sequence at carboxyl terminus of the *E. coli* DNA ligase. The V(D)J RSS dependent DNA joining activity of VDJP has been studied in detail. The purified bacterially expressed VDJP recombinant proteins are able to join the DNA strands in a RSS dependent manner. The DNA joining reaction mediated by VDJP is $Mg^{++}$ dependent, but does not require ATP or $NAD^{+}$. The substrate DNA sequence specificity of the DNA joining event has been determined to be the same as for *in vivo* V(D)J recombination. DNA fragments without RSSs, or with mutant RSS were unable to be joined by VDJP. The sequences of head to head joined DNA products corresponding to the signal joint have been determined. Although the VDJP mediated DNA joining reaction shows the same substrate DNA specificity as *in vivo* V(D)J recombination, the VDJP reaction is less precise.

FZ 327 DIRECT VERSUS INVERTED REARRANGEMENTS IN THE MURINE HEAVY CHAIN VARIABLE REGION,

Linda F. VanDyk and Katheryn Meek, Department of Internal Medicine, UT Southwestern Medical Center, Dallas,TX, 75235

**Recombination of the IgH variable region gene segments is directed by recombination signal sequences (RSS) which flank the gene segments. The RSS are composed of a palindromic heptamer, a non-conserved spacer of 12 or 23 base pairs, and an A/T rich nonamer. $D_H$-$J_H$ rearrangement occurs almost exclusively by direct rather than inverted joining. We have used PCR amplification of murine bone marrow, spleen, and thymus DNA to look for all rearrangements which could theoretically occur. We isolated various $D_H$-$J_H$ joints, including direct coding and signal joints, and hybrid $D_H$-$J_H$ joints, as well as both inverted and direct $D_H$-$D_H$ rearrangements. Despite finding rare rearrangements consistently ($D_H$-$D_H$ and hybrid joints), certain potential rearrangements were never found, specifically, those having a $D_H$ segment which had rearranged at the 5' RSS while retaining the 3' RSS. These findings suggest that the observed direct rearrangement results from a recombination bias for utilization of the 3' RSS in $D_H$ rearrangement. While previous data using artificial substrates showed only a small bias for direct joining, other recent work comparing the $D_H$ 3' and 5' RSS showed a strong bias for use of the 3' RSS, yet no study to date has shown the 1000-fold preference seen in vivo. We are currently using artificial substrates both in transient and stable transfectants to recapitulate the in vivo bias and to define the responsible DNA sequences and context.**

FZ 328 DJH JOINING IN SCID MICE,

Gillian E. Wu, Jacqueline L. Pennycook, Robert A. Phillips and Yen Hui Chang. Department of Immunology, University of Toronto, Toronto, Ontario M5S 1A8 CANADA.

The "SCID" mouse is characterized by a lack of B and T cells. This defect is believed to be a consequence of a defective recombinase system. Previous research has shown that coding joints of recombination substrates are greatly aberrant, while signal joints remain relatively unaffected. Our lab has devised a quantitative PCR assay whereby *scid/scid* and normal bone marrow (BM) DJH rearrangements can be examined. The assay detects any rearrangements of DH to JH that retain the 5' recombination signal sequence (RSS) of the DH gene segment (which is the 5' primer), and the sequences immediately 3' of JH4 (the 3' primer). Rearrangements with any deletions within the DH and JH gene segments will be amplified as long as the two primer sites are retained. Amplification produced four discrete bands which were similar in size to the control C.B-17 BM DNA, but about 10 times less frequent, corresponding to DJH1, DJH2, DJH3 and DJH4 rearrangements. Structures were cloned, sequenced, and compared to similar structures from the C.B-17 BM. The *scid/scid* sequences were potentially functional joins. The average number of deletions were greater than those of C.B-17 but there were far fewer "n" insertions. The implications of these results will be discussed.

*Ig Isotype Switching*

FZ 329 REGULATION OF THE IgM PENTAMER/HEXAMER RATIO AT THE LEVEL OF POLYMER ASSEMBLY,

Joseph W. Brewer, Troy D. Randall, and Ronald B. Corley. Department of Immunology, Duke Medical Center, Durham, NC 27710

Antibodies of the IgM isotype are the first to be secreted in a primary immune response and are the most prevalent type involved in T cell-independent responses. Classically, IgM has been described as a pentameric molecule consisting of five $H_2L_2$ monomeric subunits and one joining (J) chain. Recently, it has been shown that a variety of B cell lines make IgM hexamers as well as pentamers. Hexamers contain an additional $H_2L_2$ monomeric subunit and lack J chain. Increases in the level of J chain transcription in responding B cells as a result of stimulation by lymphokines such as IL-2, IL-5, and IL-6 favor pentamer formation at the expense of hexamers in the secreted IgM. Functionally, hexamers are of interest since they mediate complement fixation 15 - 20 fold more efficiently than pentamers. We now demonstrate that normal B cells can make IgM hexamers as well as pentamers. Peritoneal B cells were found to secrete more hexamers than splenic B cells. This may be a result of differences in the B cell subpopulations present in these two anatomical sites, or it may reflect differences in previous antigen exposure (T-dependent vs. T-independent) of B cells from these two locations. In an effort to understand mechanisms that may regulate the type of polymeric IgM secreted by responding B cells, we are seeking to develop a clear picture of IgM assembly and secretion. We have found that IgM polymer assembly occurs by step-wise addition of HL half-monomer subunits and possibly also by step-wise addition of $H_2L_2$ monomer subunits. Polymerization into pentamers and/or hexamers occurs prior to the mid-Golgi. J chain appears to be added only to completed pentamers at this time. Once a completed polymer traverses the mid-Golgi, it is rapidly secreted from the cell. The relationship of these observations to the ability of B cells to secrete varying ratios of IgM pentamers and hexamers will be presented. (Supported by NIH grant AI31209)

**FZ 330** IL-10 INDUCES MONONUCLEAR BLOOD CELLS OF ACQUIRED-IgA DEFICIENT PATIENTS TO PRODUCE IgA, *in vitro*, Francine Brière, Jean-Michel Bridon, Christine Servet, Dominique Chevet* and Jacques Banchereau, Schering-Plough, Laboratoire de Recherches Immunologiques, Dardilly, France, *Unité de Néphrologie et Réanimation Métabolique, Hopital du Bocage, Dijon

We have demonstrated that IL-10 is a factor inducing the *in vitro* differentiation of human B lymphocytes into plasmablasts [Rousset et al. (1992), Proc. Natl. Acad. Sci. USA, 89: 1890]. In this report, we investigated the effect of IL-10 on the isotype response of mononuclear blood cells from acquired IgA-deficient patients. Among 335 adult renal transplant recipients, 4 of them developped an IgA-deficiency concommitantly to an acute viral hepatitis within 6 weeks after transplantation. When cultured *in vitro*, those cells activated through CD40 and surface Ig are not able to produce IgA when compared to mononuclear cells from normal donors. Interestingly, the blood cells of these patients were induced to secrete IgA in response to increasing concentrations of IL-10 in these culture conditions. This IL-10 induced IgA synthesis is slightly enhanced by addition of IL-2 whereas it is strongly inhibited by IL-4. This is in keeping with our previous results showing that the combination of IL-10 and TGFß induced highly purified naive B lymphocytes ($sIgD^+$ B cells) to switch to the production of IgA1 and IgA2 [Defrance et al. (1992), J. Exp. Med.,175: 671], [Briere et al. (1992) submitted for publication].

It remains to elucidate whether IL-10 acts i) directly on B lymphocytes ii) indirectly by acting on another cell population which would subsequently activate B cells. Nevertheless, the present results indicate that in acquired IgA deficiency, the B lymphocytes are functional in their ability to produce IgA.

**FZ 331** PRODUCTION AND REGULATION OF GERMLINE TRANSCRIPTS OF THE γ2a GENE PRIOR TO SWITCH RECOMBINATION. John Collins and Wesley Dunnick. Dept. of Microbiology and Immunology, Univ. of Michigan, Ann Arbor, MI 48103-0620

Transcription of switch regions before recombination suggests that switch recombination is regulated by the isotype-specific opening of switch region chromatin. We have identified germline transcripts of the murine γ2a gene in normal B cells stimulated with LPS and interferon-γ, but not in B cells stimulated with LPS alone. Germline γ2a transcripts are similarly induced by LPS and interferon-γ in 18.81 cells. These germline transcripts can be distinguished from those of the γ2b gene by probes unique to the Iγ2a exon. We have amplified these germline transcripts from both normal B cells and 18.81 by PCR and have determined the structure of two different species. A minor species (about 5% of the total) is spliced from the Iγ2a exon to the Cγ2a exon at a site homologous to that used in the γ2b gene. The donor splice site for the Iγ2a exon of a second species is 120 bp downstream. A third species, which is 200 bp larger and perhaps the major product, is being characterized. We plan to study further the interaction of interferon-γ and IL4 and their effect on the production of γ2a and γ1 germline transcripts in 18.81 cells. Finally, we are attempting to identify nuclear proteins that are interferon-γ-inducible and bind to DNA in or near the Iγ2a exon. Such γ2a-binding proteins are candidates for isotype-specific regulators of switch recombination.

**FZ 332** SEPARATION OF HUMAN B LYMPHOCYTE SUBSETS CAPABLE OF PRODUCING EITHER IgM OR IgG BASED ON THE EXPRESSION OF mp28, A NEWLY IDENTIFIED SURFACE MARKER, Cornelis de Groot, Marcel L.C.M. Mevissen, Elly Goormachtig, Ernst Lindhout and Jaap Kwekkeboom, Cellular Immunology Group, Laboratory of Cell Biology & Histology, Academic Medical Center, University of Amsterdam, The Netherlands.

Using monoclonal antibodies raised against recombinant human IL-2, we could identify a 28kDa protein, provisionally called mp28, on the surface of the human Raji and JY B cell lines. The $NH_2$-terminal amino acid sequence of mp28 has striking homology to native T cell-derived IL-2. Mp28 surface expression was also found on a variable part of tonsil B lymphocyte populations. Except for a partial relation with increased CD38 expression and decreased CD39 expression, no coincidence could be established with any of a large panel of other surface markers. Separation of $mp28^+$ and $mp28^-$ B cells using panning on anti-IL-2-coated Petri dishes and subsequent stimulation of the separated cells with Staphylococcus aureus Cowan I and IL-2 revealed that IgM production was predominantly associated with $mp28^+$ B cells and IgG production with $mp28^-$ B cells. Immortalization of the separated cells of four different donors by electrofusion to SP2/0 (murine) or F3B6 (human/murine) fusion partners and direct culture of the hybridomas under limiting dilution conditions resulted in the production of IgG by hybridomas derived from $mp28^-$ B cells (98.7% IgG/ <1.3% IgM with SP2/0; 98.9% IgG/1.5% IgM with F3B6) and IgM by hybridomas derived from $mp28^+$ B cells (63% IgM/0% IgG with SP2/0; 98% IgM/1.6% IgG with F3B6). These result indicate that the capacity to produce IgM resp. IgG was an intrinsic feature of the separated B cell subsets and not a result of selective copurification of an accessory cell type. Therefore, mp28 expression may prove to be a potent marker to discriminate between IgM and IgG-committed B lymphocytes.

**FZ 333** EFFECT OF A SV40 T ANTIGEN TRANSGENE DRIVEN BY THE γ1 GERMLINE PROMOTER ON EXPRESSION OF THE ENDOGENOUS γ1 GENE. Wesley Dunnick[1], Laura Elenich[1], Jan Berry[1], J. Latham Claflin[1], and Charles C. Chu[2]. [1]Dept. of Microbiology and Immunology, Univ. of Michigan, Ann Arbor, MI 48103-0620 and [2]Laboratory of Immunology, NIAID, Bethesda, MD 20892

Germline transcription of the switch and constant regions seems to precede switch recombination to each of the murine heavy chain genes. The cells that express these germline transcripts are rare in mice, as they arise (usually) as the result of an antigen driven immune response. We have attempted to immortalize cells expressing the γ1 germline transcript by construction of transgenic mouse lines with SV40 T antigen under the control of the γ1 germline promoter. Since they are controlled by the same promoter sequences, expression of the endogenous γ1 germline promoter and expression of T antigen should be induced in the same cells at the same time. Our hope is that T antigen will extend the number of division cycles of such cells. B cells from the T antigen mice stimulated with LPS express more γ1 protein than do B cells from normal littermates. Second, by a PCR based assay (DC-PCR) we have preliminary evidence of increased switch recombination to γ1 in the T antigen mice. Thirdly, B cells from T antigen mice stimulated with LPS express γ1 germline transcripts, whereas B cells from normal mice do not. These three lines of evidence suggest that cells in the process of switch recombination to the γ1 heavy chain gene remain in this state longer in the T antigen transgenic mice that would the same cells in normal littermates. This results in an increased number of cells expressing γ1 germline transcripts, increased switch recombination to γ1, and increased expression of γ1 heavy chains.

**FZ 334** INTERLEUKIN INDUCTION AND TISSUE SPECIFICITY OF TRANSCRIPTION OF TRANSGENIC IMMUNOGLOBULIN SWITCH REGIONS. Laura A. Elenich, Wesley A. Dunnick, Minzhen Xu*, and Janet Stavnezer*. The Univ. of Michigan Medical School, Ann Arbor, MI, and *The Univ. of Massachusetts Medical School, Worcester, MA, USA.

Splenic B cells can be induced to undergo immunoglobulin heavy chain class switching to specific isotypes by treatment with mitogens and lymphokines, and the specificity of the switch event may be mediated by differential accessibility of the constant region genes. We are studying the regulation of switching to the γ1 isotype, which is induced by treatment of splenic B cells with LPS and moderate amounts of IL-4. We have previously characterized the γ1 germline transcripts and have studied the function of the promoter region in transient and stably transfected cell lines. We are extending these studies of the promoter region using transgenic mice. Our transgene constructs contain the first 200 bp of the transcripts and 150, 1500 or 2100 bp of 5' flanking sequences. Another construct contains 2100 bp of 5' and 12 kb of 3' sequences. The 17 kb construct appears to contain all of the elements required for proper regulation, while analysis of the transgenic lines with the shorter promoter region constructs reveals that these transgenes are transcribed in splenic B cells and the thymus, as opposed to the endogenous transcripts which are only induced in B cells treated with IL-4. We are in the process of further analyzing the IL-4 inducibility and expression in various tissues of these transgenes. The expression patterns should indicate the location of elements important in the regulation of the γ1 germline transcripts.

**FZ 335** USE OF EXTRACHROMOSOMAL EUKARYOTIC DNA SUBSTRATES TO STUDY IMMUNOGLOBULIN HEAVY-CHAIN SWITCH RECOMBINATION, Doina Ganea*, Carol Lepse* and Ramesh Kumar# *Department of Biological Sciences, Rutgers University, Newark, NJ 07102, and #DNX, Princeton, NJ 08540.

Switch recombination, a DNA rearrangement event occuring during B cell development, results in the replacement of one constant region with another, following DNA deletion events due to the recombinational process. Although not site-specific, the switch recombination involves specific switch regions. Questions related to the existence and number of isotype-specific switch recombinases, as well as to the regulatory mechanisms involved in the expression of the switch recombinase(s) have been difficult to answer due to the lack of appropriate DNA substrates. Here we report on the construction of extrachromosomal eukaryotic DNA substrates to study switch recombination in murine lymphocytes. The backbone substrate was the pMC1neo PolyA plasmid which contained a neomycin resistance gene (neo) under the control of the thymidine kinase promoter (TKpr). The initial plasmid was modified by the insertion of a polylinker containing the unique ClaI, BglII, and SalI sites between the TKpr and the neo gene, and a transcriptional terminator was further cloned into the BglII site. To insure the autonomous extrachromosomal replication of the plasmid a 3.6 kb fragment containing the polyoma early region genes was cloned into the SspI site. Fragments containing the Sμ and either Sγ2a or Sγ2b regions were then cloned into the SalI and the ClaI sites, respectively. Switch recombination should result in the deletion of the transcriptional terminator flanked by the switch regions, followed by expression of the neo gene. Initial experiments in 3T3 cells indicated that the transcriptional terminator was functional, and that its activity was orientation-specific. The 18-81 pre-B cell line, which switches in vitro from μ to γ2b secretion, was electroporated with the μ-γ2b or μ-γ2a switch recombination substrates, followed by selection in medium containing LPS (10 ug/ml) and G418 (1.2 mg/ml). The number of positive wells, as well as the number of viable colonies in each well was determined, and plasmid DNA preps (Hirt's preps) were characterized by restriction enzyme (RE) analysis. The frequency of positive wells, and the average number of viable colonies per well were similar for the two DNA substrates, arguing against the existence of several different isotype-specific switch recombinases. Different RE patterns were recorded for most positive wells, suggesting the occurrence of multiple independent switch events. The majority of the events seemed to be the result of deletions involving both switch regions.

**FZ 336** A GENETIC SYSTEM TO TEST FOR RNA TRANS-SPLICING IN ISOTYPE SWITCING, James Gorman*, Suzanne Li, M.D., Ph.D., Fred Alt, Ph.D.*, *HHMI, Harvard Medical School, Childrens Hospital, Boston, MA 02115

Prior to heavy chain class switching into a given immunoglobulin (Ig) heavy chain constant region locus, the constant region locus is transcribed, producing an RNA called the germline transcript. We have established a genetic system to test whether this germline transcript functions as a substrate for an RNA trans-splicing reaction, a mechanism of RNA splicing that can produce a mature mRNA from two separate RNA molecules. This mechanism of RNA processing has previously only been proven to occur in lower eukaryotes. We are testing whether trans-splicing can connect a VDJ RNA to a Cγ2b constant region RNA, to produce a mature VDJ-Cγ2b RNA before class switching to the γ2b locus has occurred at the DNA level. A construct was designed which should preserve the trans-splicing signals on the germline transcript if these signals exist, but which fuses the germline Cγ2b gene to a gene that confers G418 resistance ($G418^R$). The transcript encoded by this construct should serve as a target molecule in a trans-splicing reaction. If a trans-splicing reaction were to occur, it would allow the expression of the mRNA for $G418^R$. This trans-spliced product should be detectable by virtue of its biological activity, its sequence, and its novel size. Transfection of the trans-splicing construct into a normal pre-B cell line confers a level of $G418^R$ similar to that conferred by a positive control $G418^R$ construct. Transfection of the trans-splicing construct into a defective pre-B line that lacks a VDJ, and therefore cannot donate a VDJ to a trans-splicing reaction, produces no such shift in $G418^R$. Further cotransfection of a productive VDJ-$C_\mu$ construct, which should provide a donor RNA substrate for the putative trans-splicing reaction, significantly increases $G418^R$ to a level equal to or greater than the positive control. The shape of the resistance curves and the design of the experiment, in which $G418^R$ is assayed without imposing prior G418 selection, suggest that the $G418^R$ is expressed by most or all cells in the population. Preliminary Southern analysis has provided no evidence for production of the hybrid RNA molecules by interchromosomal recombination. In preliminary S1 analysis, a band the size of the predicted trans-spliced product is observed. These results may be due to trans-splicing between the productively rearranged VDJ-$C_\mu$ transcript and the Iγ2b-Cγ2b'-$Neo^R$ transcript, to produce a functional VDJ-Cγ2b'-$Neo^R$ message and protein. Additional experiments are being pursued with the aim of conclusively proving or disproving the existence of RNA trans-splicing in Ig production.

**FZ 337** HIGH FREQUENCY ISOTYPE SWITCHING IN HYBRIDOMA CELLS, Polly D. Gregor, Gadi Spira and Matthew D. Scharff, Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461

Hybridoma cells spontaneously switch to downstream isotypes at frequencies of $10^{-6}$ -$10^{-7}$ depending on the cell lines and the isotype being assayed. By adapting the ELISA spot assay to quantitate switch variants, we were able to determine the exact rate of this event using fluctuation analysis. Two independent $IgG_1$-producing hybridomas were identified that switched from $IgG_1$ to downstream constant regions at rates of 4-6x$10^{-6}$ cell/generation. Within these two parental cell populations, 36-65 and PC1.4.1, clonal variants were identified that had higher rates of switching. By sequentially enriching for such clones we were able to obtain clones that had increased their rate of switching by 2-3 orders of magnitiude. In one experiment where 20 fresh subclones of such a high switch variant were analyzed, the frequency of IgG2a switch variants was between .01 and 87% with an average frequency of 16%.

Genetic analysis confirmed that switch variants isolated from 36-65 have undergone gene deletions typical of those seen following isotype switching in vivo. Interestingly, the $IgG_1$ switch region undergoes frequent and sequential deletions in PC1.4.1 subclones having a high potential for isotype switching, whereas the $IgG_{2a}$ switch regions of these same cells are uneffected.

FZ 338 STUDY OF THE CONTROL AND THE MECHANISMS OF IG GENE SWITCH RECOMBINATION USING IG HEAVY CHAIN INTRON ENHANCER DELETED MICE

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It has been widely speculated that transcription through the switch region sequence is a prerequisite for switch recombination of immunoglobulin heavy chain genes. Based on the Cre-loxP recombination system, we have developed a gene targeting method which can be used to delete RNA transcription, or DNA recombination regulatory elements in murine embryonic stem cells. We have utilized this technique to determine the role of the heavy chain intron enhancer element and the transcriptional requirements of the $\mu$ chain gene, in immunoglobulin class switch. Accordingly, we have generated a mouse strain in which the entire J chain gene cluster and the intron enhancer ($J_H$-E$\mu$) of the heavy chain gene was deleted. The analysis of switch recombination in B cells from the heterozygous mutant mice indicates that the $\gamma$1 switch region on the targeted chromosome still undergoes switch recombination as efficiently as that on the wild type chromosome. In contrast, however, switch recombination of the $\mu$ chain switch region was partially blocked on the targeted chromosome. We found that the targeted chromosome was unable to make $\mu$ chain transcripts as determined by a sensitive polymerase chain reaction assay. Thus, these results indicate that switch recombination of the $\gamma$1 immunoglobulin gene is independently controlled from $\mu$ chain switch region gene and do not rely on the heavy chain intron enhancer. Furthermore, transcription through switch region sequences may not be essential for switch recombination.

FZ 339 REGULATION OF IgA EXPRESSION, Deborah Lehman, Michael Park, Arti Pandya and Zhenling Zhang, Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia 23298

Regulation of IgA expression occurs at two levels, isotype switching to generate mIgA expressing cells and maturation of mIgA expressing cells to IgA secreting cells. The first step in the process of isotype switching is the appearance of germline transcripts consisting of sequence ($I_H$) 5' to the switch region spliced to the constant region *in lieu* of a variable region formed by VDJ joining. To elucidate the role of these transcripts in isotype switching to IgA, their regulation in response to transforming growth factor $\beta$ (TGF-$\beta$) was investigated. Previous studies demonstrated that the addition of TGF-$\beta$ to lipopolysaccharide (LPS) stimulated B cell cultures induced an increase in the steady state level of $\alpha$ mRNA that was not detectable until two days after initiation of culture. The increase in steady state level was due in part to the appearance of germline $\alpha$ mRNA transcripts. To determine if the increase in steady state level of $\alpha$ mRNA was due to an increase in rate of transcription, nuclear run-on analysis of B cells stimulated with LPS alone or with TGF-$\beta$ for 1-3 days was performed. Both I$\alpha$ and C$\alpha$ probes were used. Regardless of length of culture, there was no significant difference in hybridization to either I$\alpha$ or C$\alpha$ probes suggesting that in these cultures, TGF-$\beta$ does not increase the rate of transcription of either germline or productive $\alpha$ mRNA. Subsequent studies to determine if TGF-$\beta$ increases the stability of $\alpha$ mRNA indicated that an increase in stability alone was unlikely to account for the increase in $\alpha$ mRNA. The relatively low level of $\alpha$ mRNA in cells stimulated with LPS alone coupled with the fact that in LPS-stimulated cultures the detectable $\alpha$ mRNA consists of productive transcripts whereas in the presence of TGF-$\beta$ both productive and germline transcripts are present makes a definitive conclusion difficult. The finding that TGF-$\beta$ induced increases in $\alpha$ mRNA are not due exclusively to increased transcription suggests both that the germline transcripts themselves play a role in isotype switching and that the role of TGF-$\beta$ in isotype switching to IgA may not be analogous to IL-4 in IgE production.

FZ 340 ENDOGENOUS EXPRESSION OF A NOVEL FORM OF δ ON THE SURFACE OF WEHI-231. John G. Monroe, Helen Haggerty, and Vicky Lentz. Department of Pathology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

WEHI-231 is a murine B cell lymphoma which has been utilized extensively as a model for an immature-stage B cell, and its functional response to antigen receptor crosslinking as a model for immature B cell tolerance. This study clearly demonstrates that WEHI-231, in contrast to immature B cells, expresses d on its surface. In addition, we show here that this cell line expresses sIgM, CD5, and FcRγ, but lacks the B cell specific isoform of CD45 (B220). In contrast to crosslinking μ, crosslinking of the endogenous surface IgD on WEHI-231 was unable to generate a negative growth response in these cells. This inability may be due to uncoupling from normal surface Ig signalling pathways as this receptor also failed to elicit detectable PI hydrolysis or calcium mobilization following crosslinking by anti-δ antibodies; although induced changes in tyrosine phosphorylation were observed. Structural analysis of d on WEHI-231 demonstrates its molecular weight to be greater than that of d on BAL-17, a mature B cell lymphoma, and primary B cells. This difference appears to be primarily related to differences in N-linked glycosylation. Endogenous expression of surface IgD on WEHI-231 is inconsistent with its representing the classically defined immature-stage B cell. The structural and signalling differences associated with δ on these cells suggest the potential for developmentally-regulated d function and a model for study of sIgD signal transduction.

FZ 341 THE INDUCTION OF ISOTYPE SWITCHING FOLLOWING THE ADDITION OF ANTI-IgM TO LPS OR HEPLER T CELL STIMULATED NORMAL OR LYMPHOMA B CELLS, E. Charles Snow, Department of Microbiology and Immunology, University of Kentucky Medical Center, Lexington, KY 40536.

The addition of either anti-IgM or anti-IgD to LPS stimulated normal B cells or $BCL_1$ lymphoma cells in culture results in a dose dependent diminution in cellular proliferation. This same pattern is observed when the anti-receptor antibodies are titrated into cultures of B cells stimulated with anti-TCR activated, fixed T helper cells. Both the normal and tumor B cells secrete IgM following the addition of either LPS or the activated T helper cells. The titration of either anti-IgM or anti-IgD into these cultures is found to inhibit, in a dose dependent fashion, the secretion of IgM by both populations of B cells. However, only the anti-IgM antibodies causes the LPS or T helper cell stimulated normal or tumor B cells to switch to IgG1 secretion. One possible explanation for this finding is that the occupancy of the membrane IgM receptors expressed by $IgM^+IgD^-$ B cells, which develop as a consequence of LPS or T helper cell induced B cell expansion, initiates the mechanisms by which some B cells switch to IgG1 synthesis.

**FZ 342** ROLE OF INTERLEUKIN-6 OR SOLUBLE CD23 MOLECULE ON THE HUMAN IGE SYNTHESIS, Toshio Tanaka, Tetsuji Naka, Hiroshi Ochi, Yoshinori Katada, Masaichi Aitani, Masaki Suemura, and Tadamitsu Kishimoto, The Third Department of Internal Medicine, Osaka University Medical School, Osaka 556, JAPAN

The process of differentiation of resting B cells into IgE secreting cells has been shown to consist of three steps: (1) induction of expression of germline transcript of ε gene that are made up of Intervening sequence (Iε) and constant region (Cε), (2) actual S-Sε recombination at DNA level, and (3) induction of expression of productive transcript of ε gene (VDJ-Cε). Recently in human system antibodies to CD40, whose natural ligand is believed to be expressed on activated T cells, and IL-4 have been demonstrated to induce IgE production by purified B cells. In this process IL-4 acts as a class-switching factor by inducing expression of germline transcript of ε and signals through CD40 molecule in the presence of IL-4 provide actual S-S recombination. Moreover, several cytokines have been reported to modulate IgE synthesis by B cells stimulated with anti-CD40 plus IL-4 or by whole peripheral blood lymphocytes stimulated with IL-4. For instance, TNF-α, IL-6 or soluble CD23 molecule positively regulates IgE synthesis whereas α-IFN, γ-IFN or TGF-β has been demonstrated to inhibit IgE synthesis. Indeed, IL-6 augmented IgE synthesis and anti-IL-6 antibody inhibited IgE synthesis by purified tonsillar B cells. The production of IL-6 by B cells stimulated with anti-CD40 plus IL-4 was found. These results thus indicate that endogenously produced IL-6 may play an important role on the IgE production. To address the mechanisms through which IL-6 or other cytokines modulate IgE synthesis, the expression of germline transcript (Iε-Cε) or productive transcript (VDJ-Cε) was measured by reverse transcription (RT)-PCR or Northern blot analyses. The addition of IL-6 or anti-IL-6 antibody did not reveal any effect on the expression of germline transcript but IL-6 augmented and anti-IL-6 inhibited the expression of productive transcript of ε gene by anti-CD40+IL-4-stimulated B cells. Furthermore, anti-IL-6 also inhibited IgG production by such B cells, suggesting that IL-6 may exert its activity as a late-acting factor by inducing S-S recombination or regulating productive Ig gene expression after B cells are switched. By using recently developed technique to detect S-S recombination (digestive circularization-PCR) by Dr. Chu for mouse γ1 or Dr. Thyphronitis for human ε, we are now testing this possibility and will discuss acting points of these cytokines on human IgE synthesis.

*B Lineage Restricted Genes*

**FZ 343** JAW-1, A LYMPHOID-RESTRICTED INTEGRAL MEMBRANE PROTEIN LOCALIZED TO THE ENDOPLASMIC RETICULUM

Timothy W. Behrens, Jaya Jagadeesh, Jonathan W. Yewdell and Louis M. Staudt. Metabolism Branch, NCI and Laboratory of Viral Diseases, NIAID, NIH, Bethesda, MD 20892.

As part of a program to isolate novel lymphoid-restricted genes, we used differential hybridization to clone a novel human gene, Jaw-1, which encodes a 539 amino acid protein with two notable structural features: a 140 amino acid domain which is predicted to form a coiled-coil and is homologous to the myosin heavy chain tail region, and a hydrophobic domain located near the carboxy terminus. Examination of the mouse Jaw-1 gene revealed that these two structural motifs are highly conserved in evolution. Northern blot analysis revealed that Jaw-1 mRNA is expressed at high levels in spleen, thymus, B and T lymphoid cell lines and in a few myeloid cell lines. Jaw-1 mRNA was not detectable in non-lymphoid tissues or non-hematopoetic cell lines. Indirect immunofluorescence and confocal microscopy using an affinity purified polyclonal rabbit anti-Jaw-1 antibody showed that Jaw-1 colocalizes with the endoplasmic reticulum (ER) protein BiP in murine lymphocytes. Furthermore, in HeLa cells transfected with the full length Jaw-1 cDNA, Jaw-1 colocalized with another ER protein, PDI. Removal of the carboxy terminal hydrophobic domain of Jaw-1 resulted in its expression in the cytoplasm suggesting that this hydrophobic domain functions as a transmembrane domain. In vitro translation of Jaw-1 cDNAs in the presence of pancreatic microsomes resulted in a tight association of Jaw-1 protein with microsome membranes which could not be extracted by pH 11.5 Na Carbonate or 1M KCl, again suggesting that Jaw-1 is an integral membrane protein. Interestingly, however, Jaw-1 has no amino-terminal signal sequence. When inserted in intact microsomes, the entire Jaw-1 protein is sensitive to digestion by trypsin suggesting that Jaw-1 has a Type II orientation with the myosin homology region oriented towards the cytoplasm. Thus, the Jaw-1 protein is a lymphoid-restricted ER membrane protein which is oriented in a manner which might allow it to interact with cytoplasmic coiled-coil proteins.

**FZ 344** THE IDENTIFICATION OF GENES SELECTIVELY EXPRESSED IN MURINE PLASMACYTOMAS

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Using a novel PCR strategy we have constructed a murine plasmacytoma minus B lymphoma subtractive cDNA library and probe. We have further characterized subtractive cDNAs that are expressed in most murine plasmacytoma cell lines, but at a low level, if at all, in B lymphoma cell lines. Expression in normal plasma cells has been assessed in two systems: **1)** splenocytes cultured *in vitro* for 5 days with LPS; and **2)** spleens or lymph nodes from mice reconstituted with hematopoietic stem cells infected with an IL6 producing retrovirus. On the basis of this characterization, some subtractive cDNAs provide markers of the normal plasma cell phenotype and others are associated with the malignant plasmacytoma phenotype.

**Markers of normal plasma cells**

Of six genes in this category, three were expressed also in most pre-B lymphoma cell lines. By sequence two of these genes were identified as **placental alkaline phosphatase** and **syndecan**. Although syndecan was previously shown to have this "on-off-on" pattern of expression, there are no reports of alkaline phosphatase expression in pre-B cells. The other three genes were not expressed in pre-B lymphomas, although they were expressed at a low level in some of the B lymphoma cell lines. **XLR-3** is a new member of the XLR multigene family. **EGP314** is a pan-epithelial glycoprotein with homology to a basement membrane adhesion molecule. **PC315** expression is restricted to the B lineage and is inducible by IL6.

**Markers of malignant plasma cells**

Two genes were not up-regulated in IL6- or LPS-stimulated splenocytes. They are both expressed at high level in most plasmacytomas, but are not detectable in B or pre-B lymphomas. **PC251** is a new member of the hematopoietic growth factor receptor superfamily with closest homology to the IL5 receptor α. It is expressed at a uniform low level in all normal tissues. **PC326** is a new member of the ß transducin repeat family of proteins (other members of which are transcriptional repressors). It is also expressed in testis, and has a dysregulated pattern of expression in somatic cell hybrids between a plasmacytoma and either T cells or L cells.

**FZ 345** ANALYSIS OF THE STRUCTURE AND METHYLATION PATTERN OF THE HUMAN mb-1 GENE, Peter D. Burrows, and Hyunjung Ha, Department of Microbiology, Division of Developmental and Clinical Immunology, University of Alabama at Birmingham, Birmingham, AL 35294

A 5.7 kb EcoRI genomic DNA fragment encoding kthe B cell specific human mb-1 gene has been cloned and sequenced in its entirety. The mb-1 protein is coded by five exons; the first exon (112 bp) contains the 5' untranslated region and most of leader sequence, and is separated by 1.6 kb from the second exon containing the remaining (17 bp) leader sequence and most of the extracytoplasmic sequence (300 bp). The third exon, separated by 239 bp from the second exon, contains the remaining (50 bp) extracytoplasmic and transmembrane sequence (66 bp) and the first amino acid (3 bp) of the intracytoplasmic region. The remaining intracytoplasmic and 3' untranslated sequences are encoded by exon 4 (69 bp) and exon 5 (504 bp), which are separated by 126 bp. The overall structure of the human mb-1 gene is very similar to the murine mb-1 gene, including the number and approximate size of exons. The promoter region of the mb-1 gene lacks a TATA element but contains two EBF (Early B cell factor) binding motifs and a weak consensus sequence, 5'-PyPyCAPyPyPyPyPy-3' identified in several mammalian TATA-containing genes. The first intron contains a potential NF-κB binding site (GGGGCTTCCC). A long stretch (~120 bp) of AG rich sequence downstream of an Alu repeat sequence that contains a potential strong stem-loop structure, was identified between exon 3 and exon 4, and another Alu family repeat (~310 bp) was identified in the 3' flanking region. An examination of the pattern of methylation of HpaII/MspI restriction enzyme sites around the 5' flanking region showed a striking correlation of demethylation with cell type-specific transcription of the mb-1 gene. One of the hypomethylated HpaII/MspI sites is located 8 bp downstream of the putative NF-κB binding motif, suggesting a role for this transcription factor in the regulation of human mb-1 expression. (Supported by NIH Grants AI30879 and CA13148; Dr. Burrows is a Leukemia Society Scholar)

**FZ 346** A NOVEL FORM OF PHOSPHOCHOLINE INDUCES PROTECTION AGAINST *STREPTOCOCCUS PNEUMONIAE* INFECTION IN X-LINKED IMMUNODEFICIENT (XID) MICE. Randy T. Fischer*, Gretchen Guelde+, Dan Longo* and James J. Kenny+, *Biological Response Modifiers Program and +Program Resources, Inc./DynCorp, NCI-FCRDC, Frederick, MD 21702. Mice expressing the recessive X-linked immune deficiency gene (*xid*) are highly susceptible to infection with *S. pneumoniae* because they are unable to respond to the immunodominant phosphocholine (PC) epitope or other thymus independent type 2 (TI-2) antigenic components of the cell wall and capsule of this pathogen. We have previously shown that the group I, PC-specific B cells, which produce protective anti-PC antibodies in normal mice, are clonally deleted in *xid* mice via a receptor-mediated process that appears to be antigen-driven. Since passive transfer of anti-PC antibodies protects *xid* mice from infections with *S. pneumoniae*, it was of interest to induce a PC-specific response in these mice using a thymus dependent (TD) form of PC. Immunization of BALB/c mice with the traditional TD form of PC, diazophenylphosphocholine coupled to keyhole limpet hemocyanin (DPPC:KLH), protects these mice from challenge with *S. pneumoniae* but is not able to generate protective antibodies in *xid* mice. A new TD form of PC, 6-O-(phosphocholine)hydroxyhexanoate coupled to KLH (EPC:KLH), induced PC-specific, T15-id$^-$ antibody responses primarily of the $IgG_1$ isotype which provided complete protection against challenge doses 1,000-10,000 fold over the $LD_{50}$ for *xid* mice. The majority of the antibody response to EPC:KLH remains PC-specific even after multiple immunizations, whereas DPPC-KLH induces mainly group II, PPC-specific antibodies following 2° challenge. Antisera from the EPC:KLH immunized *xid* mice have the ability to bind to encapsulated *S. pneumoniae* as demonstrated by flow cytometric analysis (FCM). Antisera from *xid* mice immunized with DPPC:KLH were not able to bind *S. pneumoniae*. PC-specific hybridoma antibodies derived from EPC:KLH immunized *xid* mice were capable of passively protecting *xid* mice to $10^4$ CFU/mouse. These results indicate that by modifying the PC-carrier linkage used for immunization, a protective PC-specific immune response can be induced in immunodeficient *xid* mice.

**FZ 347** ANALYSIS OF CD45 EXPRESSION ON UNSTIMULATED B CELLS OR B CELLS STIMULATED WITH EITHER LPS OR IL5, Karen S. Hathcock, Hiroyuki Hirano, Shinya Murakami, and Richard J. Hodes,Experimental Immunology Branch, NIH, Bethesda, MD 20892

In this study, we examined the expression of CD45 molecules on murine B cells that were either unstimulated or were stimulated with LPS, or with the T helper cell cytokine IL5. Flow cytometric analysis of cell surface expression of CD45 epitopes revealed that unstimulated and stimulated B cells expressed equivalent amounts of total CD45 but that activation with rIL5 induced a $CD44^{hi}$, hyaluronate-adherent subpopulation of B cells that expressed a markedly altered pattern of expression of exon-specific CD45R or B220 determinants. The predominant CD45 immunoprecipitated from either unstimulated or LPS-stimulated B cells was of the high molecular weight form (approximately 220 kDa) usually associated with B cells. In contrast, CD45 immunoprecipitated from the hyaluronate-adherent subpopulation of IL5-activated B cells was predominantly lower molecular weight forms. PCR analysis of amplified CD45 cDNA showed distinct expression profiles characteristic of each B cell population studied. The highest molecular size PCR product, corresponding to expression of all three variably expressed CD45 exons (A, B, and C) was dominant in resting B cells and in LPS-activated B cells but was selectively reduced in hylauronate-adherent IL5-activated B cells, where lower molecular size PCR products predominated, corresponding to expression of one or two of the variable exons. Further, all B cell populations expressed a strong lower molecular weight PCR product corresponding in size to the product expected when exons A, B, and C are spliced out of CD45 mRNA. Changes in CD45 expression were also observed in activated $BCL_1$ tumor cells, demonstrating in this monoclonal B cell population that changes in CD45 isoform expression could be induced. Thus, alternative splicing of CD45 mRNA, as well as cell surface expression of CD45, is altered in a highly selective fashion by specific mediators of B activation, providing a novel parameter for analyzing the functional differences of B cells activated by diverse stimuli.

**FZ 348** IDENTIFICATION OF NOVEL LPS-RESPONSE GENES IN B CELLS THROUGH THE USE OF A GENE-TRAP VIRUS AND FACS-GAL. William G. Kerr, Mark Heller and Leonard A. Herzenberg, Dept. of Genetics, B-007, Beckman Center, Stanford University, Stanford, CA 94305.

We had developed a gene-trap retrovirus (Gensr1) that activates expression of a FACS-selectable reporter gene, *E. coli lacZ*, upon integration within a cellular gene (*CSHSQB* 54: 767 '89; *Methods* 2: 261 '91). If the virus integrates in an intron of an expressed gene, splicing of the *lacZ* exon to an upstream cellular exon can occur, resulting in a gene fusion between a cellular gene and *lacZ*. We have confirmed this model, since cDNA cloning and sequencing of eight independent *lacZ* fusion mRNAs indicates that cellular exon(s) are fused to the splice-acceptor site of *lacZ*.

Bacterial endotoxin or lipopolysaccharide (LPS) can induce the maturation of B-lineage cells at several points along the differentiation pathway. As an approach to identifying genes which play a key role in the ordered maturation of B-lineage cells, we have attempted to identify genes restricted to specific stages of B cell differentiation. To achieve this goal, we have infected B-lineage cell lines with the Gensr1 virus, FACS-selected β-gal+ clones, and identified clones where *lacZ* expression is altered by LPS-stimulation. Northern blot analysis demonstrates that clones where β-gal activity is altered by LPS treatment contain *lacZ* fusion mRNAs whose abundance is affected by LPS stimulation. We have obtained cDNA clones for several LPS-regulated *lacZ* fusion mRNAs via the RACE procedure. Similarity comparisons of these exons with the GenBank database indicates that they are novel sequences. Northern blot analysis with one of the LPS-response cDNAs suggests that it may be expressed in a stage-specific manner during B cell differentiation. We are currently determining when these novel LPS-response genes are expressed in B cell differentiation as well as whether they are expressed in other tissues which respond to LPS. These results and further structural analysis of these genes will be presented.

**FZ 349** REGULATED EXPRESSION OF IMMUNOGLOBULIN SECRETION SPECIFIC mRNA IN B-CELL HYBRID LINES, C. Milcarek, M. Hartman, and S. Matis, Department of Molecular Genetics and Biochemistry, University of Pittsburgh, School of Medicine, Pittsburgh, PA. 15261

We have shown that *in vivo*, sequences downstream of the secretion-specific polyadenylation site play an important role in the preferential selection of the Ig sec polyA site in plasma cells [Lassman et al., J. Immunol. **148**:1251-1260 (1992) and Lassman & Milcarek, J. Immunol. **148**:2578- 2585 (1992)].

To determine if mRNA half-life might also play a role in determining the sec:mb Ig mRNA ratio at steady state, somatic cell hybrids were made between a ouabain-resistant activated/memory B-cell (A20.2J $Ou^R$, secretory: membrane mRNA ratio of 2:1) expressing the mouse gamma 2a Ig gene and a HAT-resistant mouse plasma cell tumor (J558L) which lacks an endogenous heavy chain, but produces mRNA ratio of 20:1, sec:mb on tranfected Ig genes). We find that the plasma cell phenotype is dominant at the mRNA level because the hybrids express high levels of the gamma 2a Ig mRNA and a sec:mb ratio of 36:1. When we measure the half-lives of the gamma 2a Ig mRNAs in the hybrids, we find that there was a two-fold increase in message life time relative to that seen in the activated/memory B-cell parent, but, this increase is seen for both the sec and mb species. We therefore conclude that differential half-life does not account for the changes in sec:mb ratio in the hybrids.

When we look *in vitro* at polyadenylation competent nuclear extracts prepared from the murine oubain:HAT resistant hybrids (plasma cell like) versus the oubain-resistant (memory cell like) parent, we see cell line specific qualitative and quantitative differences in the proteins which are UV cross linked to the input Ig mRNAs substrates. Some of these proteins may be responsible for the differential use of the Ig sec polyA site seen *in vivo*.

**FZ 350** INDUCTION OF 5-LIPOXYGENASE IN MANTLE-ZONE B-LYMPHOCYTES AT ACTIVE SITES OF INFLAMMATION, Vickie R. Shannon, and Michael J. Holtzman, Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110

The process of B cell activation and differentiation may be regulated at a molecular level by the products of phospholipid-arachidonic acid metabolism, and in particular, by the products of arachidonate 5-lipoxygenase. Thus, the determination of biochemical mechanisms for B lymphocyte biosynthesis and degradation of phospholipid-arachidonate metabolites is a critical step towards understanding the biologic function of lymphocytes. We have utilized immunochemical and enzyme assay techniques to characterize peripheral blood and tissue lymphocyte 5-lipoxygenase expression under normal conditions and during conditions of inflammation. Our immunohistochemical studies of tissue lymphocytes in the colon and lung using anti-5-lipoxygenase antibodies and indirect biotin-avidin-peroxidase detection demonstrate that, in contrast to tissue from normal colon or lung in which 5-lipoxygenase antigen was confined to neutrophils and macrophages and was absent in lymphocytes, the same tissues involved by granulomatous disease (Crohn's disease of the colon or tuberculous disease of the lung) exhibit markedly positive immunostaining with anti-5-lipoxygenase antibody throughout the mantle zone of lymphoid aggregates. Specificity for 5-lipoxygenase immunostaining was verified by immunostaining with three different anti-5-lipoxygenase antibodies, antigen competition experiments with synthetic 5-lipoxygenase peptide (for anti-5-lipoxygenase peptide antibody), and lack of immunostaining with anti-12/15-lipoxygenase antibodies. Our results provide initial evidence for selective increase in lymphocyte lipoxygenase in inflammatory disease and suggest a possible role for the 5-lipoxygenase products in B-cell recruitment and activation.

**FZ 351** ***HIS50, a rat homolog to mouse J11d/Heat Stable Antigen, and human CD24; cDNA isolation and sequence*** T.S.K. Tong, S.V. Hunt* and D. Opstelten, Department of Biochemistry, University of Hong Kong, Hong Kong and *Sir William Dunn School of Pathology, University of Oxford, U.K. *(This work is supported in part by grants from the Croucher Foundation, Hsin Chong - K.N. Godfrey Yeh Eduation Fund and Sino-British Fellowship Trust Fund, Hong Kong.)*

The mouse monoclonal antibody HIS50 (isotype:IgM) recognizes glycosylphosphatidylinositol(GPI)-linked surface molecules present on B lymphocytes and their precursors in rat bone marrow but absent from plasma cells (Hermans et al., in preparation). We obtained structural information on HIS50 antigen through isolation of HIS50 cDNA by expression cloning in COS. A cDNA library from rat late pre-B tumour cells was constructed using the CDM8 shuttle vector (complexity about $5x10^5$). After three rounds of screening of transfected COS using magnetic beads coated with HIS50, a 472bp cDNA encoding HIS50-binding epitopes was isolated. The two XbaI fragments were each subcloned for DNA sequencing by the chain termination method. For each fragment the sequence was read from both T3 and T7 promotors, yielding sequence information that overlapped by at least 120 bp. The full sequence revealed an open reading frame encoding 76 amino acids residues (aa) with a predicted N-terminal signal peptide of 26 aa and a predicted GPI-displacing region of 23 aa. In the predicted mature peptide (the remaining 27 aa), half of the aa are potential sites of glycosylation (9 O-linked and 4 N-linked). A search in the EMBL data base, version 12/91, revealed that HIS50 cDNA is homologous to two other GPI-linked molecules: murine Heat Stable Antigen M1/69-J11d (HSA)(90% in 471bp overlap) and human CD24 (70% in 416bp overlap). With respect to HSA both N- and C- terminal peptides were 96% homologous, while the mature peptides were 82% homologous. With respect to CD24, the N-terminal peptides were 92% , the C-terminal peptides 61% and the mature peptides 45% homologous. The most striking common features are (1) their short length (27 to 31 aa residues) (2) their abundance of potential sites of O-linked and N-linked sugar side chains (3) their GPI-anchorage. The function of such short and probably highly glycosylated surface molecules is unknown. They may play a role in hemopoiesis, since the expression of all three is largely confined to hemopoietic cells. However there are major differences in the types of hemopoietic cells expressing these molecules. The cloned cDNA will be used for further studies on the function of HIS50 antigen.

**FZ 352** ISOLATION AND CHROMOSOMAL MAPPING OF THE HUMAN IMMUNOGLOBULIN-ASSOCIATED B29 GENE, William J. Wood, Jr.,[1] Alexis A. Thompson,[2] Julie Korenberg,[3] Xia-ning Chen[3], William May[2], Randolph Wall[1] and Christopher T. Denny[2,4], Department of Microbiology and Immunology, UCLA School of Medicine,[1] Department of Pediatrics,[2] Department of Genetics, Cedars-Sinai Medical Center,[3] Department of Microbiology and Molecular Genetics,[4] University of California, Los Angeles, Los Angeles, California 90024

The B29 gene encodes a B cell specific membrane protein in the immunoglobulin antigen receptor complex. B29 is a crucial member of this receptor complex and is believed to function as an effector of signal transduction in a manner analogous to the CD3 components of the T cell antigen receptor. We have isolated a full length human B29 cDNA clone by using a murine B29 cDNA probe. We show that there is an extremely high degree of evolutionary conservation between the human and mouse proteins, particularly in the transmembrane and intracytoplasmic regions where the identity is 96%. In addition, the intracytoplasmic region in both proteins contains an identical peptide motif which is present in a number of molecules involved in lymphocyte activation. Genomic Southern blot analysis of human cell lines hybridized with both murine and human B29 cDNAs give patterns consistent with a single copy gene occupying a small region of the genomic sequence. Using human B29 cosmid DNA, we have localized the B29 gene to human chromosome 17q23 via fluorescence in situ hybridization. B29 is the the first gene localized to this area of the genome. A subset of human B cell leukemias are found to have karyotypic abnormalities in this same region of chromosome 17. We propose a role for B29 in the malignant phenotype of leukemic cells.

*Regulation of B Lineage Gene Expression*

**FZ 400** CONSTITUTIVE AND CELL CYCLE-REGULATED EXPRESSION OF C-*MYB* mRNA IS RELATED TO THE STATE OF DIFFERENTIATION IN MURINE B-LYMPHOID TUMORS.

Timothy P. Bender, Katrina M. Catron and Charles R. Toth. Department of Microbiology, University of Virginia, Charlottesville, VA. 22908.

We have previously shown that the steady-state level of c-*myb* mRNA is differentially regulated in murine B-lymphoid tumors. Pre-B cell lymphomas contain 10 to greater than 100 fold more c-*myb* RNA than B cell lymphomas and plasmacytomas. This difference appears to be maintained primarily by a block to transcription elongation (attenuation) which occurs in the first intron of the gene. We have recently investigated the low level of c-*myb* mRNA expression in the $BCL_1$ B cell lymphoma using amino acid starvation to block $BCL_1$ cells in a $G_0/G_1$ state or in combination with aphidicolin to prevent progression into S-phase. Starved cells express little detectable c-*myb* mRNA. However, upon release into the cell cycle the level of c-*myb* mRNA increases 7 - 10 fold during late $G_1$/early S-phase as measured by DNA synthesis. Aphidicolin does not inhibit the increase in c-*myb* mRNA expression indicating that this increase is independent of DNA synthesis. We have used elutriation to compare expression of c-*myb* mRNA in other murine B-lymphoid tumor lines. Interestingly, the level of c-*myb* mRNA in pre-B cell lymphomas is not cell cycle regulated indicating that a switch from constitutive to a cell cycle related mode of expression may occur during B cell development. We have recently mapped the site of attenuation, using nuclear run-on assay, to a 300bp restriction fragment in the first intron of the gene. This fragment lies in a region of DNA sequence that is highly conserved (84%) between mouse and human. We detect four types of protein nucleic acid interactions in this region: 1) a protein/DNA interaction more readily detected in cells expressing low levels of c-*myb* mRNA, 2) three protein/DNA interactions detected equivalently in all cell types, 3) a protein/DNA interaction detected more readily in cells containing large amounts of c-*myb* mRNA and 4) a protein/RNA interaction detected equivalently in all cell lines tested. The protein/DNA interaction detected in cell lines expressing low levels of c-*myb* mRNA appears to involve a member of the NF-kB/c-*rel* family of DNA binding proteins. This interaction is inhibited by purified $I_kB$ and the binding site involves a novel DNA sequence. We are presently assessing the potential role of these protein/nucleic acid interactions in attenuation.

**FZ 401** FUNCTION OF NFHB(BSAP) BINDING SITES IN IgH GENE EXPRESSION, Barbara K. Birshtein, Fang Liao, Paul Rothman* and Mallika Singh, Dept. of Cell Biology, Albert Einstein College of Medicine, Bronx, N.Y. 10461 and *Dept. of Medicine, Columbia U. Coll. P&S, New York, N.Y. 10027

We have defined several binding sites within and 3' of the IgH gene cluster for a B lineage-specific DNA binding protein-NFHB. Through cross-competition of EMSA, footprint analysis, UV cross-linking and site-specific mutagenesis, we have inferred that NFHB is most likely identical to a B cell-specific transcription factor, BSAP (encoded by *Pax-5*). NFHB(BSAP) is present in pro-B, pre-B and B cell lines and absent from plasma cell lines. Recent experiments have shown that an NFHB binding site upstream of the $C_\alpha$ gene is identical to that required for induction by LPS and IL4 of $\epsilon$ germline transcripts in a B cell line. Furthermore, an NFHB binding site from the region 5' of $S_\gamma 2a$ can substitute for the $\epsilon$- associated NFHB site. These results indicate that NFHB(BSAP) is a non-isotype specific protein required for $\epsilon$ germline transcript induction.

NFHB(BSAP) binding sites in the 3'$\alpha$ enhancer (3'$\alpha$E) have been shown by EMSA to interact with the predominant B lineage-specific DNA binding protein for this regulatory element. Experiments assessing DNAseI hypersensitive sites and methylation show that the 3'$\alpha$ region is inaccessible at early stages of B cell differentiation when NFHB is present, and becomes accessible at the plasma cell stage when NFHB is no longer detectable. Site-specific mutagenesis of the NFHB binding site in 3'$\alpha$E shows, as expected, no deleterious effect on its activity in plasma cell lines. However, in B cell lines where 3'$\alpha$E is inactive, the mutated 3'$\alpha$E has a transcriptional enhancement equivalent to the $\mu$ intron enhancer. The inverse relationship between 3'$\alpha$E activity and NFHB implies that NFHB acts as a repressor for 3'$\alpha$E . We infer that 3'$\alpha$E becomes active during the transition from B to plasma cells, thus making it a candidate for involvement in processes that occur during this transition, such as somatic mutation, class-switching, and shifts in production from membrane-bound to high levels of secreted antibody.

**FZ 402** DEVELOPMENTALLY CHANGING CHROMATIN STRUCTURE AT THE KAPPA IMMUNOGLOBULIN 3' ENHANCER, Veronica C. Blasquez, Patricia A. Smith, Julie A. Wilberding, and Shar'ron E. Harris, Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556.

Two transcriptional enhancers have been identified within the mouse kappa immunoglobulin locus: the intronic enhancer (Ei) that occurs between the Jk and Ck gene segments and the 3' enhancer (E3') which is located 8.5 kb downstream of the gene. To understand the role of the 3' enhancer in the activation of the kappa immunoglobulin locus, we have analyzed its chromatin structure in a number of cultured B-cell lines arrested at various stages of differentiation. Utilizing the indirect end-labeling technique, we identified 100-bp of E3' to be DNAse I hypersensitive in pro-B-cell chromatin. This altered structure was B-cell specific and Ei was found to be concurrently hypersensitive at this early B-cell stage. The E3' hypersensitive site was developmentally stable through the pro, pre-, and mature B-cell stages. Interestingly, a dramatically changed pattern of DNAse I hypersensitivity was observed in antibody-secreting plasma B-cells which exhibited spreading of nuclease susceptibility to regions upstream and downstream of the initial E3' site. These results correlated with changes in the methylation state of a proximal MspI/HpaII site. The role of nuclear factors in the formation of these DNAse I hypersensitive sites was investigated by gel retardation analysis. Experiments aimed at establishing the role of the observed E3' structural transition in plasma-B cells are under way.

**FZ 403** GENOMIC STRUCTURE AND CHROMOSOMAL MAPPING OF THE MURINE CD40 GENE, Debra Cockayne, Chris Grimaldi, Raul Torrest, Edward Clarkt and Maureen Howard, DNAX Research Institute, Palo Alto, CA 94304 and †University of Washington, Seattle, WA 98195

The B cell-associated surface molecule, CD40, is likely to play a central role in the expansion of antigen-stimulated B cells, and their interaction with activated helper T cells. In the present study we have isolated genomic clones of murine CD40 from a mouse liver genomic DNA library. Comparison with the murine CD40 cDNA sequence revealed the presence of 9 exons that together contain the entire murine CD40 coding region, and span approximately 16.3 kd of genomic DNA. The intron/exon structure of the CD40 gene resembles that of the low affinity nerve growth factor receptor gene, a close homolog of both human and murine CD40. In both cases the functional domains of the receptor molecules are separated onto different exons throughout the genes. Southern blot analysis demonstrated that murine CD40 is a single copy gene that maps in the distal region of mouse Chromosome 2.

FZ 404 OCT-2 IS CRITICAL FOR HIGH LEVEL IMMUNOGLOBULIN PRODUCTION BY B CELLS AND FOR POSTNATAL SURVIVAL, Lynn M. Corcoran[1], Maria Karvelas[1], G.J.V. Nossal[1], Zheng-sheng Ye[3], Tyler Jacks[2] and David Baltimore[3]. [1]The Walter and Eliza Hall Institute for Medical Research, P.O.Royal Melbourne Hospital, Victoria 3050, Australia, [2]*Cancer* Research Center, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02142, U.S.A., and [3]The Rockefeller University, 1230 York Avenue, New York, NY10021-6399, U.S.A.

Oct-2 is a transcription factor of the POU box family that is believed to play some role in the B cell-restricted expression of immunoglobulin (Ig) genes. It is expressed predominantly within the B cell lineage, and has binding sites in all Ig gene promoters and some enhancers. Oct-2-deficient mice, generated through gene targeting, survive to birth but no further. Mutants die within hours of birth, without having suckled, but displaying no obvious anatomical abnormality. B cell precursors are present in lymphoid tissues of mutant animals in numbers and with characteristics similar to those of normal littermates, and numbers of $IgM^+$ B cells in neonatal liver and spleen are only slightly reduced. However, B cells from mutant animals have a marked defect in their capacity to secrete Ig upon mitogenic stimulation in vitro. Thus, Oct-2 may play its most critical role in mature B cells by promoting high level transcription of productively rearranged Ig genes in response to mitogenic or antigenic stimulation.

FZ 405 TGFβ INHIBITS AN EARLY EVENT IN LPS AND PHORBOL ESTER INDUCED κ TRANSCRIPTION, Michael A. Damore, Sidne A. Omori, Cindy Sue Malone and Randolph Wall, Molecular Biology Institute and Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA 90024

We previously reported that transforming growth factor *beta* (TGFβ) inhibited bacterial lipopolysaccaride (LPS) activation of immunoglobulin κ light chain gene transcription in pre-B cells without detectably affecting activation of NF-κB binding. We now have determined that the LPS-induced CAT activity of the 474 bp κ intron enhancer is inhibited to the same extent as the endogenous κ genes in TFGβ–treated pre-B cells. This establishes that the κ intron enhancer is the primary target of TGFβ inhibition. Next, we analyzed other LPS-incuced transcription factors known to bind in the κ intron enhancer for sensitivity to TFGβ. TGFβ had no detectably effect on the LPS-induced increase in Oct-2 binding, but it completely blocked κBFA activation. The LPS-stimulated activity of a variant κ enhancer containing a mutated κBFA motif was recuced relative to the wild type enhancer but this altered enhancer still retained sensitivity toTFGβ suggesting that other enhancer motifs are also affected by TGFβ. We carried out kinetic studies to establish the timing of TFGβ inhibition of κ induction. We found that TGFβ blocked a very early event in both LPS- and phorbol myristate acetate- (PMA-) activated κ gene transcription. Since κBFA activation is observed later in LPS induction (i.e., at 4 hours following LPS stimulation), we conclude that the inhibition of κBFA only partially accounts for the effects of TGFβ on κ gene activation and that the inhibition of a still unidentified transcription factor activated earlier in induction is likely to be the main cause of the inhibitory effect of TGFβ.

FZ 406 INTERFERON-γ INDUCED IRG-47 GENE EXPRESSION AND REGULATION, Michael Gilly and Randolph Wall, The Molecular Biology Institute and Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA 90024

The IRG-47 gene product is predicted to encode a GTP-binding protein whose mRNA is rapidly and transiently induced in pre-B, B- and fibroblast cell lines in response to interferon-γ (IFN-γ). Like many other genes induced by the interferons, the IRG-47 gene is rapidly activated at the transcriptional level following induction. We investigated the gene control sequences mediating the induction, by either deletion or site-directed mutagenesis, followed by reintroduction of these as CAT reporter constructs into responsive cell lines by transient transfection. The promoter of the IRG-47 gene is quite simple and is composed of overlapping IRS and IRF-1/2 motifs upstream of a TATA box. Interestingly, the region upstream of the IRG-47 promoter located between 450 and 900 bp 5' of the TATA box exerts a strong negative regulatory influence on the minimal promoter domain. Removal of this 5' region results in a very strong constitutive promoter activation upon transfection into 3T3 fibroblasts. This constitutive activity approximated the maximal level of IRG-47 expression in IFN-γ induced cells. These observations suggest the IRG-47 gene is principally regulated through the removal of negative control, rather than by the induction of positive-regulatory mechanisms. Consistent with the prediction that all of the positive-acting transcription factors required for IRG-47 induction must pre-exist within the cell before IFN-γ induction, IRG-47, like primary response genes, is IFN-γ induced in the presence of cyclohexamide. Distinct induction kinetics and requirements for new protein synthesis characterize IFN-γ inducible IRG-47 mRNA activation in 70Z/3 pre-B versus L929 fibroblasts. Furthermore, different mechanisms appear to control the induction of this gene in different cell types.

FZ 407 HUMAN λ IMMUNOGLOBULIN LIGHT CHAIN TRANSCRIPTIONAL REGULATION, Michele A. Glozak and Bonnie B. Blomberg, Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, FL 33136.

We have recently reported the localization of the first transcriptional enhancer in the human lambda (λ) immunoglobulin light chain locus (Blomberg et al, J.Immunol 147:2354, 1991). Enhancer activity was contained on a 1.2 kb SstI fragment, with partial activity retained on a core 111 bp PstI-SstI fragment. This enhancer is located 11.7 kb downstream of Cλ7, the most 3' lambda constant region gene recently identified and described by our laboratory to be expressed in normal lymphocytes (Bauer and Blomberg, J.Immunol 146:2813, 1991). The enhancer is orientation independent, tissue specific (active in B cells but not in T cells), and independent of NF-κB. Using a CAT assay system, we have since determined that the complete enhancer resides on a 311 bp PstI-SstI fragment. This 311 bp fragment is 2-4 fold as active as the 111 bp PstI-SstI fragment in a human B cell line. We also have preliminary evidence for a developmental stage specific negative element 3' of the 311 bp fragment, since removal of this flanking region allows the activity of the enhancer in a pre-B cell line. The human λ enhancer shares 72-74% identity with the murine λ enhancers. By using electrophoretic mobility shift assays and methylation interference analysis, we have identified a site that is unique to the human λ enhancer to which a protein factor (Human Enhancer Lambda Protein) from human B cells binds. The murine λ enhancers do not contain this site. Competition studies indicate that the factor(s) which bind the HELP motif also weakly bind another enhancer element 5' of the HELP motif. We are currently cloning the HELP binding protein(s), to determine the role the protein(s) plays in the expression of human λ immunoglobulin light chain genes.

FZ 408 IDENTIFICATION OF LYMPHOID SPECIFIC ELEMENTS WITHIN THE IMMUNOGLOBULIN HEAVY CHAIN 3' ENHANCER. Patrick Grant and Sven Pettersson. Karolinska Institute, Center for Biotechnology, Novum, 141 57 Huddinge, Sweden.

The B lymphocyte specific immunoglobulin 3' heavy chain enhancer contains multiple nuclear protein binding sites. We have shown that this enhancer can be dissected into three functional domains, two of which are lymphoid specific in their activity. Removal of the octamer sequence from the entire enhancer results in a drop in activity to only 60% of wild type activity in transient transfection assays and an octamer deleted enhancer remains lymphoid specific in its activity. Deletion analysis and DNase I footprinting have identified several additional lymphoid specific Ets-like motifs involved in the regulation of the 3' enhancer. At least one of these motifs can be bound by an *invitro* translated member of the Ets-domain family of proteins. We will present data on the involvement of Ets-domain proteins on the tissue restricted expression of the IgH 3' enhancer.

FZ 409 THE $DQ_{52}$ REGION OF THE IGH LOCUS HARBORS A STRONG, TISSUE SPECIFIC PROMOTER, Andreas H. Kottmann and Georges Köhler, Department of Molecular Immunology, Max-Planck Institute for Immunobiology, Freiburg, Germany

The first manifestation of activated immunoglobulin (Ig) loci in the course of B-cell differentiation is the transcription of D-elements. Among the D-elements of the Ig heavy chain (H) loci of man, rat and mouse the single member family forming $DQ_{52}$ element is peculiar: This element is more strongly conserved among the species than it is to the other D elements within the same species, the $DQ_{52}$ element of mouse is transcribed prior and after rearrangement, and is used predominantly in initial D-$J_H$ rearrangements.

We previously characterized the $DQ_{52}$ DNA region to be a complex cis element interacting with several ubiquitiously and tissue specifically expressed transacting factors. Now we have investigated the promoter of the $DQ_{52}$ element. A 400 bp DNA fragment could be identified that, in conjunction with the heavy chain intron enhancer, confers tissue specific expression to an heterologous reporter gene in transient transfection studies. This fragment overlaps with the previously described DNA interaction domain. The $DQ_{52}$ promoter is as strong as a $V_H$ promoter (i.e. "Sp6") and 10 fold stronger than the promoter of the Dsp2-2 element. The concentration of binding sites for nuclear factors close to the $J_H$ proximal $DQ_{52}$ element and the strength of the newly discovered promoter is remarkable and suggests a specific role of this region in regulating IgH gene expression. To elucidate the function of the $DQ_{52}$ region during ontogeny of B cells in vivo we are in the process of generating mutant mice that have deleted the $DQ_{52}$ element and surrounding DNA by homologous recombination in embryonic stem (ES) cells.

FZ 410 IDENTIFICATION OF A NEW MEMBER OF THE IMMUNOGLOBULIN KAPPA LIGHT GENE FAMILY WHICH WAS CLONED USING AN ENHANCER-TRAP FOR GLUCOCORTICOID-REGULATED GENES, Stephen S. Lippman, John R. Bracamontes, Jacqueline Howard-Bedford, Department of Medicine, Division of Endocrinology, University of Arkansas for Medical Sciences, Little Rock, AR 72205

We have developed an enhancer-trap strategy to clone additional genes that are down-regulated by glucocorticoids in AtT-20 cells which have not been identified by other methods that are dependent on information about the protein expressed by each gene. This strategy uses the plasmid, pA10-NEO, which contains the selectable marker gene, amino-glycoside 3' phosphotransferase II, coupled to an enhancerless SV40 promoter. When transfected, this gene is only effectively expressed when integrated near an active enhancer. We have previously shown that in a stable, transformed clone of AtT-20 cells (IDG8) transfected with pA10-NEO, both NEO mRNA level and transcriptional rate is down-regulated by dexamethasone. A EMBL3 Phage clone containing the NEO gene and approximately 15 kilobases of both 5' and 3' flanking genomic DNA has been partially restriction-mapped and sequenced. A region homologous to the full kappa light-chain gene constant domain has been sequenced and found to be 68% homologous to the sequence of the functional kappa gene expressed in mouse B lymphocytes and greater than 99% homologous to the sequence expressed in human cells. Southern Analysis of genomic DNA from mouse myeloma lines (derived from the Balb/c strain) and from LAF mouse liver DNA showed a hybridizing fragment distinct from the previously known single mouse kappa gene. Polymerase chain amplification of genomic DNAs from IDG8 cells, mouse myeloma cells, and LAF mouse liver all produced the same ~500 BP fragment with primers selected to amplify only sequence derived from this new domain. The characterization of these cloned sequences will allow direct analysis of the mechanism of glucocorticoid down-regulation of this gene in AtT-20 cells. The hormone-regulated, enhancer-trap cloning of this gene suggests that glucorticoids may directly regulate transcription of immunoglobulin-superfamily genes and that these genes may be expressed in corticotrophs.

FZ 411 TRANSCRIPTIONAL REGULATION OF THE MURINE B29 GENE, Sidne A. Omori and Randolph Wall, Department of Microbiology and Immunolgy and The Molecular Biology Institute, UCLA School of Medicine, Los Angeles, CA 90024.

The B29 gene encodes the B-cell specific protein that is found in a heterodimeric complex with the mb1 gene product. This complex is associated with membrane bound immunoglobulin and is responsible for both the translocation of immunoglobulin to the cell surface as well as signal transduction in a manner analogous to the CD3 complex associated with the T-cell receptor. Unlike the mb1 gene which is turned off at the plasma cell stage, the B29 gene is expressed at all stages of B-cell differentiation. We have previously reported that B29 transcription is initiated at multiple start sites, and the upstream region directly proximal to the start sites lacks a TATA box and contains a functional octamer site. To identify DNA sequence elements necessary for controlling B29 gene transcription, a 5' deletion series of a 1200 basepair genomic DNA fragment containing sequences directly upstream of the B29 transcriptional start sites was generated. This deletion series was inserted in front of the CAT gene and tested in transient transfection assays in lymphoid and nonlymphoid cell lines. The results of these transfections reveal that there are three active regions within this 1200 basepair fragment which contribute to B29 expression: one region containing an apparent negative regulatory activity, one region with enhancer-like activity, and the region 130 basepairs proximal to the B29 multiple start sites that functions as the minimal promoter. Linker scanning mutations were done over this 130 basepair region to further identify important regions in the promoter. These mutations indicate that there are three areas within the promoter that are important to promoter function. Interestingly, the B29 gene does not appear to share any transcriptional control motifs with the mb1 gene even though these two genes are covalently heterodimerized, and their co-expression is required for the surface appearance and activity of the mIg associated complex.

**FZ 412** ANALYSIS OF THE HUMAN CD19 PROMOTER, Agostino Riva, Gaye-Lynn Wilson, and John H. Kehrl, Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

CD19 is a B lymphocyte cell-surface protein that is expressed early in B cell ontogeny and present throughout B cell differentiation until the plasma cell stage when it is lost. The CD19 gene was isolated from a human genomic library and a 5.0 kb fragment containing the 5' region subcloned into pSP65. 1200 nucleotides were sequenced upstream of the major transcriptional starting sites. No TATA box was identified, however, 2 BSAP binding sites were found immediately upstream of the start sites (similar to Kozmik et al., MCB 12, 2662, 1992). CD19-CAT constructs containing approximately 200, 400, 600, 800 or 1000 nts. upstream of the major start sites were made. A human B cell line, HS-Sultan, was transiently transfected with the CD19-CAT constructs and CAT activity measured 48 hrs later. A 14-fold induction of CAT activity over the background was observed with the 200 bp construct and nearly similar level of activity was detected with the 400 bp construct, however, all the other constructs were inactive. Five CD19 negative B-cell lines were isolated following treatment of HS-Sultan cells with methanesulfonic acid ethyl ester, negative selection three times using anti-CD19 dynabeads, FACS sorting for low levels of CD19, and cloning at 0.3 cells per well. Northern blot analysis revealed a marked reduction or absence of CD19 mRNA in the CD19 negative cell lines when compared to the parent cells. Flow cytometric analysis of the cell lines for other B cell markers failed to identify any marked change in their expression. Transient transfection of the 200 and the 400 bp CD19-CAT constructs into the 5 cell lines and Jurkat cells (also CD19 negative), surprisingly, revealed high levels of CAT activity in 2 of the cell lines and in Jurkat cells. The other 3 cell lines were inefficiently transfected, thus, no conclusions could be drawn from them. These results suggest that within the first 200 bp of the CD19 promoter are elements which can direct high levels of transcription in both B and T cells. The dramatic decrease of CAT activity beyond - 400 suggests the presence of negative regulatory elements in that region. The location of those elements which specify B cell specific expression of CD19 remain to be elucidated.

**FZ 413** POSITIONAL AND SPATIAL REQUIREMENTS OF TRANSCRIPTION FACTOR BINDING SITES WITHIN THE KAPPA IMMUNOGLOBULIN INTRON ENHANCER, Judith T. Schanke and Brian G. Van Ness, Institute of Human Genetics, University of Minnesota, Minneapolis, MN 55455.

A species comparison of the kappa immunoglobulin intron enhancer region reveals a high level of conservation of the spatial organization of the transcription factor binding sites suggesting the conserved spatial arrangement of the binding sites may be essential for full enhancer function. We are examining the importance of the positional and spatial organization of the transcription factor binding sites within the kappa immunoglobulin intron enhancer. The affects of the spacing changes were assessed by a V kappa promoter driven CAT reporter gene. Enhancer driven expression vectors were constructed with insertions between individual factor binding sites. The effects of the insertion of either 25 bases or a series of 6, 9, 16, 20 and 25 bases between each two consecutive binding sites of the intron enhancer were assessed. Both LPS responsive pre-B cell and plasma cell lines were transfected with the wild-type and modified enhancer constructs. The importance of the positions of individual binding sites within the enhancer were also examined. In order to change the relative positions of factor binding sites, individual motifs were independently mutated. An oligonucleotide containing the corresponding wild-type factor binding site was then inserted downstream of the mutated enhancer. Enhancer driven expression vectors were then transfected into cell lines representing various stages of B cell development. Results will be presented which indicate significant positional and spatial requirements of the transcription factor binding sites within the kappa immunoglobulin intron enhancer.

**FZ 414** THE HUMAN Iα1 AND Iα2 PROMOTERS: POSSITIVE AND NEGATIVE REGULATORY ELEMENTS CONTROL THE B CELL SPECIFIC EXPRESSION OF Cα1 AND Cα2 GERM LINE TRANSCRIPTS. Paschalis Sideras and Lars Nilsson . Dept. of Applied Cell and Mol. Biology, Umeå University, 901 87 Umeå, Sweden.

Treatment of human B lymphocytes with mitogens and TGF-β induces expression of germ line Cα1 and Cα2 transcripts and class switching to these two isotypes. In order to characterise further the molecular mechanism by which TGF-β and mitogens regulate the expression of the human IgA isotypes we have characterised the promoter elements that ere responsible for the transcriptional activation of their corresponding germ line genes using transient expression assays. We report here that both in the Iα1 and in the Iα2 regions, maximal promoter activity TPA and TGF-β responsiveness can be conferred by 327 bp fragments spanning the transcription initiation sites and a phylogenetically conserved region that has been previously identified. The expression of these 327 bp fragments is not restricted to the B cell lineage since in addition to the B cell lines Raji and DG75 they are also active in the myeloid cell line K562. Preliminary mutational analyses have demonstrated the importance of sequences that contain a putative CREB binding site for TGF-β and PMA responsiveness and putative PU-1 and Sp1 binding sites for basal promoter activity. Upstream distal elements that could modulate negatively the expression of these promoters, particularly in non B cells, have been identified. At least three such elements were mapped in the Iα1 and Iα2 regions. The action of these elements should presumably contribute to the B cell specific character of the Iα1 and Iα2 promoters. The Iα1 and Iα2 promoters were found to be functionally indistinguishable from each other regarding their basal level of expression and their responsiveness to TGF-β. The implications of these findings to the molecular mechanism regulating isotype switch in humans is further discussed

**FZ 415** IN VIVO FOOTPRINTING AND GEL SHIFT ANALYSIS OF THE HUMAN CD20 PROMOTER, Claire Thévenin, Brian P. Lucas, and John H. Kehrl, Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

The CD20/B1 gene encodes a B cell specific protein involved in the regulation of B cell proliferation and differentiation. Studies with 5' deletion CD20 promoter-CAT constructs have previously revealed two regions of the promoter between bases -186 and -280 and bases -280 and -454 which contain positive regulatory elements. The major sequence element present in the proximal region has been identified as a diverged octamer binding site which is important in the high constitutive expression of CD20 in B cells and in the induction of CD20 in pre-B cells. This sequence element was referred to as the BAT box and was efficiently transactivated by an Oct-2 expression vector when placed upstream of a heterologous promoter. The deletion of the BAT box in one of the CD20 promoter-CAT construct resulted in a 70% loss of activity. Mobility shift assays using probes spanning the distal region of the promoter revealed 3 DNA binding proteins, 2 present only in B cell nuclear extracts, the last one found in nuclear extracts from T cell lines, a promonocytic cell line U937, and Hela cells. Comparison of the nucleotide sequence between -454 and -280 with other promoters and enhancers of B cell specific genes revealed homologies between bp -359 and -344 with the elements kE2, μE3 and μE4 present in Ig promoters and enhancer. In vivo footprinting of this region demonstrated the interaction of 3 proteins present in B cells which interact with nucleotide sequences at positions -284/-319, -340/-353, and -450/-465. These corresponded to sites where DNA binding proteins were identified by gel shift assays. Further analysis using in vivo methylated DNA from different cell lines are in progress in order to determine the cell type specificity of these protein-DNA interactions in vivo. Deletion mutants containing 5' deletion of the CD20 promoter between -454 and -280 have been generated in order to determine the functional activity of these binding sites. The further study of the CD20 promoter should allow the delineation of the regulatory factors which specify tissue and stage specific expression of this gene.

**FZ 416 A B CELL-SPECIFIC DNA-BINDING PROTEIN COMPLEX CONTAINS A TOPOISOMERASE II,** **Carol F. Webb, Kenton L. Eneff, and Fred H. Drake[1], Immunobiology and Cancer, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, and Department of Cellular Biochemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406[1].**

**We previously identified two DNA-binding protein complexes that bound 5' of an immunoglobulin heavy chain promoter in an inducible fashion after treating cells with interleukin 5 and antigen. Both of these complexes appeared to be B cell-specific and to contain similar proteins as determined by mobility shift assay. The more 5' DNA sequence to which these proteins bound functioned as a nuclear matrix attachment site in vitro, while the more promoter proximal sequence was required for increased immunoglobulin mRNA levels in response to antigen and interleukin 5. We have used antibodies to the p170 and p180 isoforms of topoisomerase II to demonstrate that a topoisomerase II is a component of both of these B cell-specific complexes. The p180 isoform has been suggested to play an important role in transcription while the p170 isoform is thought to be directly involved in replication. Addition of antibodies that recognize both toposiomerase II isoforms in mobility shift assays caused the formation of supershifted complexes. Further studies with antibodies specific for each isoform indicated that the B cell-specific complex contained a topoisomerase II that was more closely related to the p180 isoform than to p170. Further studies to determine whether this topoisomerase II is identical to the p180 isoform or whether it is a novel form of topoisomerase II are in progress. To our knowledge, this is the first report where a topoisomerase II related protein participates in a stable, B cell-specific mobility-shifted complex. Our data suggest an hypothesis whereby immunoglobulin transcription may be increased in response to interleukin 5 and antigen by relief of torsional stress along the gene.**

*Clonal Deletion vs. Anergy vs. Activation*

**FZ 417** REGULATION OF SURFACE IMMUNOGLOBULIN EXPRESSION IN TOLERANT B CELLS, Sarah E. Bell & Christopher C. Goodnow, Department of Microbiology & Immunology, & Howard Hughes Medical Institute, Stanford University, CA 94305.

In a number of experimental models, tolerance to self antigens has been shown to be mediated by elimination or functional inactivation of self-reactive lymphocytes. The precise events involved in this process, however, are still obscure. Functional silencing of auto-reactive B cells as a result of encountering a critical level of autologous antigen *in vivo* has been documented in double transgenic mice expressing a rearranged anti-hen egg lysozyme immunoglobulin gene, and a gene construct encoding lysozyme. The tolerant state in B cells in these mice appears to be closely linked to a profound and long-lasting downregulation of membrane IgM, but not IgD antigen receptor, implying that tolerance may be mediated by changes in the antigen receptor complex itself. It is intriguing why IgM shows this downmodulation and not IgD. To investigate this phenomenon, we are studying the involvement of Ig-associated molecules, namely the product of the mb-1 and B29 genes. The reduced surface expression of IgM in these cells is not reflected at the mRNA level, since the expression of mb-1, B29, and $\mu$ appears normal, suggesting that regulation of expression may be post-translational. In metabolic labelling studies, less IgM accumulates in the double transgenic cells, the majority not being processed to an endoglycosidase H-resistant form. This suggests that IgM may be selectively targeted for degradation in the endoplasmic reticulum. The biosynthesis and assembly of Ig and associated molecules in relation to the function of the antigen receptor in tolerant cells will be discussed.

**FZ 418** OVERLAP OF THE ANTI-SM AND ANTI-DNA RESPONSES IN MRL/lpr MICE. Deborah Bloom, Jean-Luc Davignon, Marc Retter, Mark Shlomchik, David Pisetsky, Phil Cohen, and Robert Eisenberg and Stephen Clarke. Departments of Microbiology and Immunology and Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, and Duke University Medical Center, Durham, NC, and the Fox Chase Cancer Center, Philadelphia, PA 19111.

We have examined spontaneous autoimmunity to the Sm antigen in MRL/Mp-lpr/lpr mice. This response is found in 25% of MRL/lpr mice at 5 months of age and occurs at a similar frequency in patients with systemic lupus erythematosus. We have sequenced the expressed VH and $V_K$ genes from 41 anti-Sm hybridomas obtained from 9 anti-Sm positive MRL/lpr mice. We found that the response in individual mice was oligoclonal, and that there was evidence of restriction in VH and JH gene use, and possibly of $V_K$ use. Evidence of selection by the Sm antigen was equivocal. Surprisingly, however, several features suggest selection by a second antigen, DNA. 1). Over half of the 41 anti-Sm hybridomas bound ssDNA and some also bound dsDNA; 2). Hybridomas selected for Sm binding overlapped significantly in VH gene use with those selected for binding to DNA. 3). Anti-Sm hybridomas that bound Sm and DNA, but not those that bound only Sm, had a high content of VhCDR3 arginine residues; 4). Somatic mutation in some clones was responsible for increasing antibody affinity for ssDNA and the acquisition of dsDNA binding. This overlap in the anti-Sm and anti-DNA responses may have implications for the activation and selection of anti-Sm and anti-DNA B cells in MRL/lpr mice.

**FZ 419** CONTACT DEPENDENT ACTIVATION OF IMMATURE B CELLS: ROLE OF T CELL DERIVED SIGNALS AND THEIR MODULATION BY LIGATION OF B CELL SURFACE MOIETIES. Robert Brines, Jhagvaral Hasbold and Gerry G.B. Klaus. Laboratory of Cellular Immunology, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.

Fixed, anti-CD3 pre-activated T cell lines, or membranes prepared from them, can stimulate resting B cells from neonatal mice to proliferate. We have used this system to determine the influence of T cell derived signals on immature B cells. Maximal T cell membrane co-stimulation was expressed 6-12 hours after activation with insoluble anti-CD3. Neonatal B cell activation was most marked in the presence of Th2 cells, however, significant proliferation was also seen with Th1 cell lines. Antibodies to IL-4, but not IL-2 or IL-5, partially blocked co-stimulation by Th2 but not Th1 derived T cell membranes. T cell dependent B cell activation in this system was not MHC restricted as proliferation was equally marked in the presence of H-2 incompatible, activated T cells. However, antibodies specific for the I-A but not the I-E class II molecule were able significantly to inhibit B cell proliferation, suggesting a possible signalling function for I-A molecules during early B cell development. Ligation of surface IgM (sIgM) or IgD (sIgD) during the first 24 hours of co-culture blocked B cell proliferation in a dose dependent manner, whereas inclusion of anti-$\mu$ or anti-$\delta$ 48 hours after initiation of the co-culture had no effect on proliferation. This is in marked contrast to mature B cells which are not inhibited by anti-$\mu$ or anti-$\delta$ under these conditions. Taken together these data show that after CD3 mediated stimulation T cells can deliver at least one signal, distinct from IL-4, capable of activating immature resting B cells in an MHC-unrestricted fashion. An interaction between CD40 on B cells and its ligand expressed on activated T cells is being studied as an initial, obligatory signal for immature B cell activation. Moreover, we show that negative signals can be delivered via sIgM and sIgD early during the process of T cell dependent neonatal B cell activation, suggesting that tolerance induction may still be possible during this phase.

**FZ 420** DELETION AND RESCUE OF BONE MARROW B CELLS, Rita Carsetti, Georges Köhler and Marinus C. Lamers, Department of Molecular Immunology, Max-Planck-Institut für Immunbiologie, D-7800 Freiburg, FRG

We have studied the induction and maintainance of tolerance in the B-cell compartment of Ig transgenic mice. Neonatal and adult mice transgenic for IgM or IgM and IgD anti-TNP were injected with TNP-modified carriers and their B-cell compartment was compared to the B-cell compartment of PBS-treated littermates. Antigen-mediated crosslinking of membrane-bound IgM caused apoptotic cell death of bone marrow and peripheral B cells both in neonatal and adult transgenic mice with κ and μ trangenes. In mice that carried an additional δ trangene sIgD interfered with tolerance induction and protected B cells from deletion. We took advantage of the fact that in the bone marrow all stages of B cell differentiation are represented to identify the phenotype of the cell that was sensitive to deletion. sIgM+, HSAdull, B220bright cells were deleted if they expressed only IgM (in μκ mice) but were protected by coexpression of IgD (in μκδ mice). sIgM+, HSAbright, B220dull cells (immature phenotype) were never affected. In non-transgenic mice sIgM+, HSAdull,B220bright cells coexpress IgD. sIgD is absent in the immature HSAbright,B220dull pool. We conclude that B cells are sensitive to tolerance induction (i.e.deletion) at the transition from immature (HSAbright, B220dull) to mature (HSAdull,B220bright) when they are bright for IgM but have not yet acquired IgD.

**FZ 421** B CELL TOLERANCE IS MEDIATED BY ANTIGEN RECEPTOR DESENSITIZATION

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We have explored the fate of tolerant B lymphocytes using a model in which transgenic mice whose B lymphocytes express high levels of membrane immunoglobulin specific for hen-egg lysozyme are mated with mice expressing soluble hen-egg lysozyme. The resulting double transgenic mice have near normal numbers of lysozyme-specific B lymphocytes which are unable to proliferate or differentiate into plasma cells following antigen exposure. To explore the molecular basis for the non-responsive state of these tolerant B lymphocytes we have established in vivo and in vitro assay systems in which T cell help is provided by allogenic bm12 T cells. In this system the production of antigen-specific plasma cells from tolerant B cells is inhibited 100-1000 fold in response to T cell help plus antigen as compared to non-tolerant cells. Importantly, non-tolerant B cells require both T cell help plus antigen for the production of antigen-specific plasma cells, thus the defect seen in tolerant B cells could reflect alterations in the response to antigen or the ability to respond to T cell help. Two of the important signals provided by activated T cells to B cells are ligation of the CD40 antigen via the CD40 ligand and production of the B cell growth factor IL-4. Treatment of tolerant B cells with either of these stimuli in vitro resulted in responses which are indistinguishable from normal non-tolerant B cells. In contrast to the normal response of tolerant B cells to T cell derived signals, antigen receptor signalling is profoundly altered. In particular tolerant B cells fail to exhibit detectable activation of protein tyrosine kinases following antigen binding. More distal activation events such as intracellular calcium accumulation, cell cycle entry, and mitogenesis also fail to occur in antigen-activated tolerant B cells. The defect in antigen receptor signalling is selective, antigen binding induces the expression of some activation antigens on tolerant B cells (eg. CD44) but fails to induce the expression of others (B7/BB1). These data support a model whereby B cell tolerance is maintained via desensitization of the antigen receptor complex and emphasize the importance of B cell antigen receptor signalling during the immune response to T cell-dependant antigens.

**FZ 422** Tolerance and Autoimmunity In Anti-DNA Transgenic Mice

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A hallmark of many autoimmune diseases is the presence of autoreactive antibodies. We have developed a transgenic (tg) model system to examine the development and expression of B cells specific for a disease associated self-antigen, DNA. Using multiple sets of tgs we have been able to follow the fate of various kinds of anti-DNA reactive B cells in both autoimmune and non-autoimmune mice. We find that in non-autoimmune strains tolerance to DNA is manifested in different ways. In the case of the anti-ssDNA tgs, B cells with this specificity dominate the repertoire but appear blocked in their ability to secrete antibody. Their capacity to function *in vitro* as antigen presenting cells, however, appears intact. Using tgs that code for an anti-double stranded (ds) DNA antibody we see a different mechanism of B cell tolerance operating: anti-dsDNA B cells are deleted or have drastically down regulated their surface immunoglobulin receptor. When the tgs are crossed onto the autoimmune MRL/lpr genetic background both anti-ss and anti-dsDNA tg antibodies are detected. These results support the model that the presence of autoreactive antibodies in disease results from a breakdown in their regulation.

The Vh3H9 heavy chain only tg mice provide a model for surveying the potential range of specificities that result from pairing Vh3H9 with the endogenous light chain pool and for investigating how the B cells bearing these different antibodies are regulated. In a non-autoimmune background the light chain repertoire that is expressed with the Vh3H9 tg is highly restricted. The nature of this restriction suggests active editing of anti-dsDNA B cells. On the autoimmune background of the MRL/lpr, however, a different light chain repertoire is expressed with the Vh3H9 tg: there is a reemergence of light chains that are commonly found in anti-DNA antibodies. Strikingly there is also evidence of clonal expansion of a subset of autoreactive B cells, suggesting that these mice maybe responding to a specific self antigen.

**FZ 423** ANTISENSE *C-MYC* OLIGONUCLEOTIDES PREVENT GROWTH INHIBITION OF MURINE B-CELL LYMPHOMAS BY ANTI-μ OR TGF-β. Gavin Fischer and David W. Scott. Division of Immunology, University of Rochester Cancer Center, Rochester, NY, USA 14642

CH31 and WEHI-231 have been used as models for B-cell tolerance and lymphoma cell cycle regulation. We have previously shown that crosslinking of surface IgM by anti-μ or addition of TGF-β to CH31 or WEHI-231 cells leads to growth arrest and apoptosis of these cells. The *retinoblastoma* gene product (pRB) of the growth arrested cells has been shown to be in the underphosphorylated, that is, active form. We recently found that the addition of antisense oligonucleotides for *c-myc* would allow the continued growth of these lymphomas in the presence of the inhibitory concentrations of anti-μ or TGF-β. Antisense *myc* treatment also blocked apoptosis induced by both inhbitors and allowed the appearance of the (hyper)phosphorylated form of pRB, typical of log phase growing cells. Studies with delayed addition of antisense *c-myc* to centrifugally elutriated cells identified the critical period for negative signal transduction as mid- to late $G_1$. Since the addition of anti-μ to non-elutriated lymphoma cells causes mRNA levels of both *c-myc* and *c-fos* to rise within two hours and then decrease to or below baseline levels, we determined whether antisense for *c-fos* (or *c-jun*) modulated growth inhibiton by anti-μ or TGF-β. In contrast to the results with *c-myc*, addition of antisense oligonucleotides to either *c-fos* or *c-jun* did not afford protection to anti-μ-mediated growth inhibiton. Antisense oligonucleotides for *c-myc* specifically decrease the levels of the myc protein, thus suggesting that myc can act as a negative regulator of lymphoma cell growth and implying that one ultimate target is the pRB protein. (Supported by ACS grant IM-495C).

**FZ 424** ARRESTED DEVELOPMENT AND CELL DEATH DURING THE ELIMINATION OF SELF-REACTIVE B LYMPHOCYTES, Suzanne B. Hartley*, Michael P. Cooke*, David Fulcher#, Alan W. Harris@, Suzanne Cory@, Antony Basten# and Christopher C. Goodnow*
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In transgenic mice, self-reactive B lymphocytes are eliminated if they encounter membrane-bound self antigens during their development within the bone marrow. Two separate and sequential events, arrested development and cell death, bring about B cell elimination. Developmental arrest is an early outcome of antigen binding in immature B cells, blocks acquisition of adhesion molecules and receptors important for B cell migration and responsiveness, and is rapidly reversible by removal of antigen. Death of the arrested B cells occurs as a second event within one to three days, and can be delayed by expression of a *bcl*-2 transgene which enables large numbers of self-reactive B cells to escape from the bone marrow but fails to override the developmental arrest. These findings define a mechanism for B cell elimination which may be important at several stages in B cell repertoire development, and the reversible nature of the arrested stage may contribute to the breakdown of self-tolerance in *bcl*-2 transgenic mice and other examples of autoimmunity.

**FZ 425** EFFECT OF ANTI-μ ON pRB PHOSPHORYLATION AND CELL CYCLE PROGRESSION IN SYNCHRONIZED WEHI-231 B-CELL LYMPHOMAS. Luc F. Joseph and David W. Scott, Division of Immunology, University of Rochester Cancer Center, Rochester, NY 14642 USA

The product of the *retinoblastoma* gene, pRB, is the prototype of a class of tumor suppressor genes. Phosphorylation of the pRB protein is modulated during specific phases of the cell cycle and cellular differentiation. That is, pRB becomes phosphorylated in multiple stages during cell cycle entry and progression and has the properties of a cell cycle regulatory element. We have previously shown that anti-μ or TGF-β causes cell cycle blockade and apoptosis of WEHI-231 B-lymphoma cells. In such arrested cells, pRB was preferentially in the underphosphorylated (active) form, in contrast to hyperphosphorylated pRB in control, growing cells. Since anti-μ crosslinking during early $G_1$ is critical for cell cycle arrest, we have examined the state of phosphorylation of pRB in synchronized WEHI-231 cells treated with anti-μ or TGF-β. Using centrifugal elutriation, we now have evidence that anti-μ crosslinking of membrane IgM, or the addition of TGF-β to these cells, prevents the phosphorylation of the *retinoblastoma* gene product at mid- to late $G_1$ and leads to arrest at the $G_1$/S border. Moreover, there seems to be a critical point in late $G_1$ at which membrane IgM crosslinking is no longer effective at preventing pRB phosphorylation. These results suggest that the phosphorylation state of pRB is the cause, rather than the result of, cell cycle arrest in these B-cell lymphomas. (ACS grant IM-495C and USPHS grant F31 GM15005.)

**FZ 426** REGULATION OF CELL CYCLE PROGRESSION BY ANTI-μ IN HUMAN B-CELL LYMPHOMAS: ROLE OF RB AND *MYC*. Sally C. Kent and David W. Scott, Division of Immunology, University of Rochester Cancer Center, Rochester, NY 14642

Crosslinking of surface IgM receptors by μ chain specific antibodies leads to growth arrest and apoptosis in a subset of murine B-cell lymphomas. These cells undergo a critical signaling event in early $G_1$ and are blocked in their progression from $G_1$ into S. In such inhibited cells, pRB, the growth suppressor product of the *retinoblastoma* gene, is predominantly in the underphosphorylated or active form and *c-myc* transcription in dramatically inhibited. Our laboratory has now found that similar events occur in two anti-μ growth inhibitable human Burkitt lymphoma lines, Ramos (B-lymphocyte like) and Daudi ($EBV^+$ lymphoblastoid), but not in the anti-μ resistant Raji Burkitt lymphoblastoid cell line. Thus, crosslinking of surface IgM on Daudi and Ramos for 20-24 hours leads to growth arrest and the accumulation of the underphosphorylated or active form of pRB. Propidium iodide staining revealed significant apoptosis of Ramos cells that was apparent by 36 hours. Anti-μ treatment of Ramos also leads to depletion of both cytoplasmic and nuclear *myc* protein by 24 hours, as judged by intracellular immunofluorescence. Interestingly, intense pinpoint nuclear staining was observed in Ramos and Raji at 6 hours after anti-μ; this pattern of nuclear *myc* staining was still observable in Raji at 24 hours, at which time the staining in Ramos had disappeared. Control, untreated lymphoma cells displayed diffuse cytoplasmic and nuclear staining at all times. These studies implicate a role for the *myc* product and pRB in regulating the growth of these Burkitt lymphoma lines. (Supported by ACS grant IM-495C and USPHS grant T32 AI 07285.)

**FZ 427 ANTI-IDIOTYPE ANTIBODIES THAT SELECTIVELY IDENTIFY A RESTRICTED SUBSET OF ANTI-AChR ANTIBODIES THAT CAUSE NEUROMUSCULAR DISEASE SYMPTOMS IN EXPERIMENTAL MYASTHENIA GRAVIS.** **K.A. Krolick, P.A. Thompson, and B. Diaz. Dept of Microbiology, University of Texas Health Science Center, San Antonio, TX 78284.**

**It has long been noted that the correlation is often very poor between levels of serum antibodies against the acetylcholine receptor (AChR) and the severity of neuromuscular disease symptoms in myasthenia gravis (MG) patients. Our strategies for determining explanations for these observations have focused on the notion that only particular anti-AChR antibodies (that make up varying proportions of the total anti-AChR antibody titer from patient-to-patient) are directly responsible for causing neuromuscular dysfunction. Thus, we have examined the disease-causing potentials of each of the individual antibody species that make up the total spectrum of anti-AChR antibodies produced in a model system of MG. Serum anti-AChR antibodies produced by Lewis rats with experimental autoimmune myasthenia gravis (EAMG) were fractionated by preparative isoelectric focusing (pIEF). pIEF-purified serum fractions, each highly enriched in a small number of clonotypic species of AChR-reactive antibody, were passively transferred into healthy naive rats. Results of such transfers indicated that anti-AChR antibodies produced by Lewis rats are composed of at least two main subsets of antibody; one minor subset is capable of directly impairing AChR function, while the remaining subset is not capable of directly impairing AChR function. Additionally, a well-characterized monoclonal antibody (mAb35), as reported by other investigators, has reactivity against a "main immunogenic region" (MIR) of AChR and has potent disease-causing potential. mAb35 has been determined, by co-migration in pH-gradient gels (IEF), to have a serum equivalent in rats with EAMG. In light of its potent disease-causing character and, therefore, likely immunopathological relevance in vivo (i.e., represented in the anti-AChR response of Lewis rats with EAMG), mAb35 was used as the immunogen for the preparation of anti-idiotypic antibody reagents. One monoclonal anti-mAb35 (anti-Id) antibody has been generated which appears to be specific for a highly restricted subset of antibodies in EAMG rat serum. This anti-idiotype is currently being evaluated for use as a probe capable of differentiating between the disease-causing and the non-disease-causing subsets of anti-AChR antibodies.**

FZ 428 TOLERANCE TO NATIVE DNA CAN BE BROKEN BY IMMUNIZATION WITH DNA-PEPTIDE COMPLEXES

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Mice genetically predisposed to the autoimmune disease systemic lupus erythematosus develop autoimmunity to DNA including the production of autoantibodies specific for native, mammalian DNA (nDNA). Recent results from our laboratory as well as others have indicated that both early, IgM and the later appearing, IgG anti-DNA autoantibodies in autoimmune mice have the structural characteristics of secondary immune, selectively stimulated antibodies. Based upon the selection for particular antibody variable-region structures as the autoantibody response progresses from IgM to IgG, the most likely stimulus for this autoimmune response is native DNA or complexes containing nDNA. These findings are somewhat at odds with previous results demonstrating that mammalian nDNA either alone or in complexes with an immunogenic protein is generally not immunogenic. Likewise, mice transgenic for rearranged immunoglobulin genes that encode an anti-DNA antibody are tolerant to nDNA. In the present experiments mice were immunized with complexes of DNA and an immunogenic, DNA-binding peptide, Fus1. Mice immunized with such complexes produce IgG anti-ssDNA antibody after the second immunization and anti-nDNA antibody after the third or fourth immunization. The specificity and variable region structures of DNA-Fus1 induced monoclonal anti-DNA antibodies are similar if not identical to those of autoimmune monoclonal anti-DNA antibodies. Moreover, the immunopathological function of the induced anti-DNA antibodies is similar to those in autoimmune lupus. The difference in time interval between the appearance of IgG antibody specific for ssDNA versus nDNA may be a reflection of the time necessary for the generation of B cells specific for nDNA. This could occur either by somatic mutation of the immunoglobulin variable regions of B cells initially specific for ssDNA or the low frequency escape from tolerance of B cells initially specific for nDNA. These results suggest that tolerance to nDNA can be overcome by immunization with nDNA in an appropriately immunogenic form. Supported by NIH AI 26833, BRSG-RR05423, and AI07238.

FZ 429 MYASTHENIA GRAVIS AUTO-ANTIBODIES REACTIVE WITH A MAIN IMMUNOGENIC REGION (MIR) OF THE ACETYLCHOLINE RECEPTOR CAN CROSSREACT WITH MYOSIN AND INTERFERE WITH ITS ATPase ACTIVITY. Sumathy Mohan and Keith A. Krolick. Department of Microbiology, University of Texas Health Science Center, San Antonio, Texas 78284.

Auto-antibodies obtained from the plasma of patients with the neuromuscular disorder, myasthenia gravis (MG), were found to demonstrate reactivity against both the classic target antigen, the acetylcholine receptor (AChR), as well as muscle myosin. This observation was consistent with several previously published reports. However, it was also observed in the present study, that much of the dual reactivity contained in MG plasma, originally assumed to be due to the production of two independent antibody responses, was due instead to the ability of individual clonotypic species of anti-receptor antibodies to also bind myosin. This cross-reactivity was unexpected and particularly interesting in light of the clear dissimilarities in the overall biochemistry (structures) and functions of these two molecules. Furthermore, the cross-reactivity demonstrated by these antibodies appeared to involve a main immunogenic region (MIR) of the acetylcholine receptor alpha subunit, and an enzymatically important region in the head of the myosin heavy chain. Thus, patient antibodies that demonstrated reactivity with a synthetic peptide that contains the MIR region were found, also, to interfere with ATPase functions of myosin. Since, in conjunction with actin, enzymatic ATPase activity expressed by myosin is necessary for the driving of the contractile machinery of striated muscles, this observation may point to possible implications associated with anti-myosin reactivity with regard to mechanisms of disease induction in MG. Moreover, our results may also have potential significance with regard to the origin of the immunodominance of the MIR region of the AChR.

FZ 430 sIGM-MEDIATED SIGNAL TRANSDUCTION BY IMMATURE B LYMPHOCYTES DERIVED FROM DISTINCT MICROENVIRONMENTS, Amanda Norvell and John G. Monroe, Department of Pathology, University of Pennsylvania Medical School, Philadelphia, PA 19104

Mature sIgM+IgD+ B lymphocytes respond positively to antigen receptor cross-linking while immature B lymphocytes expressing sIgM but not sIgD are not activated by such stimulation. At different stages of mouse ontogeny, B cells arise from stem cells present in the fetal liver, neonatal spleen, and adult bone marrow. Studies have suggested that B cells from these different anatomic sites may represent distinct lineages and, therefore, may differ phenotypically and functionally, We have initiated studies to compare the molecular basis for the unresponsiveness of each of these populations in order to evaluate lineage-specific differences in antigen receptor-coupled signal transduction Our laboratory has previously demonstrated a specific signalling defect in neonatal splenic immature B cells which relates to their ability to generate intracellular biochemical second messengers upon sIgM cross-linking. This defect in the signalling pathway has been correlated with a component of the antigen receptor which is present in mature B cells but absent in the sIgM+IgD- neonatal spleen cells. In contrast, immature B cells derived from the adult bone marrow, while similarly unresponsive, do not manifest this signalling difference; but appear to be regulated later in the signal transduction process. We are currently evaluating whether fetal liver-derived immature B cells manifest the neonatal spleen or adult bone marrow signalling phenotype. In light of suggestions that these cells come from distinct lineages, comparisons of their signalling capabilities will allow as assessment of their relationship in a more functional manner.

FZ 431 B-CELL TOLERANCE IN AUTOIMMUNE PRONE MICE. Cynthia Rubio and David Nemazee. Univ of Colorado HSC and National Jewish Center, Denver, CO 80262

We have previously found a defect in clonal elimination of autoreactive B-cells in lupus prone MRL/lpr mice. This defect was demonstrated in genetic crosses between MRL/lpr mice and mice transgenic for IgM anti-MHC class I genes (3-83 Tol 1 transgenic line), in which a large clone of B-cells of defined specificity can be analyzed in vivo. The transgene-encoded antibody recognizes H-2K molecules of all allelic forms tested except for d and f. In contrast to immunologically normal mice, in a large fraction of (Tol 1 X MRL/lpr) X MRL/lpr backcross mice, in which the MRL/lpr's H-2K$^k$ molecules should induce deletion of autoreactive B-cells, 3-83-idiotype positive B-cells were detected in the peripheral lymphoid organs and many of these non-deleted B-cells were actively secreting anti-H-2K$^k$ autoantibodies. Mice from these crosses that were lpr/lpr developed lupus and lymphadenopathy indistinguishable from non-transgenic lpr/lpr littermates. These results suggested that the autoimmune MRL genetic background, but not homozygosity of the lpr gene, promotes the escape of autoreactive B-cells from deletion. Although we do not understand the basis of the lack of tolerance in the Tol 1 MRL/lpr crosses, these mice will be useful because they are amenable to rapid genetic analysis to determine what MRL genes contribute to this phenotype. We have extended this approach using another 3-83 transgenic mouse line (3-83$\mu\delta$) that expresses both the IgM and IgD forms of anti-H-2K and demonstrates much better allelic exclusion of endogenous immunoglobulin gene rearrangements than the Tol 1 line. Surprisingly, in 3-83$\mu\delta$ mice backcrossed three generations onto the MRL/lpr background, autoimmunity, lymphadenopathy, and early death were delayed and possibly abrogated. In addition, deletion of the autoreactive B-cells was complete. These results indicate that severely restricting the B-cell specificity repertoire prevents autoimmunity. It is also possible that reducing the absolute number of B-cells is responsible for the abrogation of autoimmunity. It is possible that B-cells with a low surface Ig antigen receptor density are more early spared from deletion by the action of MRL genes than B-cells bearing high levels of sIg, because the Tol 1 B-cells express substantially less sIg than the 3-83$\mu\delta$ line. These possibilities are currently being tested.

**FZ 432** ANERGIC SELF-REACTIVE B-CELLS IN THE RESPONSE TO SELF-LIKE FOREIGN ANTIGENS, Kevan M. Shokat & Christopher C. Goodnow Howard Hughes Medical Institute and the Department of Microbiology and Immunology, Stanford University, Stanford, CA 94305.

B-cell tolerance to self-antigens has been studied using transgenic mouse models, allowing the fate of specific self-reactive antibody producing cells to be followed. A transgenic mouse which expresses the hen egg lysozyme (HEL) specific immunoglobulin HyHEL-10 has been crossed with a second transgenic mouse expressing the neo-self antigen HEL. B-cells in the double transgenic offspring are not deleted but are rendered anergic. These cells are profoundly blocked with respect to proliferation and differentiation in response to normally stimulatory amounts of the self-antigen HEL. Extrapolated over the entire immune system one can imagine the formation of many "holes" in the specificity of the immunoglobulin repertoire created by self-tolerance which could be exploited by rapidly mutable viruses. One mechanism to limit these "holes" may be the recruitment of non-deleted self-reactive B-cells in response to challenge by self-like foreign antigens. Tolerant B-cells might somatically mutate their antigen specificity from self-specificity to specificity for the foreign antigen and thus break their tolerant state. In order to test this hypothesis anergic B-cells have been challenged *in vivo* with either HEL or duck egg lysozyme (DEL) each coupled to chicken gamma globulin (CGG) in CGG primed mice. The anergic cells undergo blastogenesis in response to challenge by HEL-CGG and appear to occupy germinal centers as characterized by surface marker expression. The ability of anergic self-reactive B-cells to somatically mutate and break tolerance when challenged with foreign antigen *in vivo* will be discussed.

**FZ 433** FUNCTION OF B CELLS EXPRESSING A HUMAN IGM RHEUMATOID FACTOR AUTOANTIBODY IN TRANSGENIC MICE. Helen Tighe, Pojen P. Chen, Rebecca Tucker, Thomas J. Kipps, Jean Roudier*, Frank R. Jirik+, Dennis A. Carson. Department of Medicine and The Sam and Rose Stein Institute for Research on Aging, University of California-San Diego, La Jolla, CA 93093-0663. *University of Marseille, Marseille, France. +University of British Columbia, Canada.

It has been suggested that resting B lymphocytes expressing IgM rheumatoid factor (RF) play a role in the afferent arm of an immune response by capturing and presenting antigen trapped in circulating immune complexes. Testing of such a theory would be facilitated by the availability of uniform populations of RF precursor B cells. We have generated transgenic mice which express the IgM heavy chain and kappa light chain genes coding for a human IgM RF, Les. The transgenic B cells display characteristics previously hypothesized to be associated with RF B cells in humans. These cells are present in relatively large numbers in the absence of high levels of serum IgM RF. The RF B cells localize to the primary B cell follicles and mantle zone regions of secondary follicles in the spleen. As hypothesized they are highly efficient antigen presenting cells for antigen complexed with human IgG, and proliferate well in response to aggregated but not monomeric human IgG. Although the levels of circulating RF are low and can not be induced under normal circumstances, RF production can be upregulated 200 fold by breeding the transgenic mice onto the autoimmune MRL/lpr background. These results strongly suggest that a major function of normal, resting RF B cells is unrelated to antibody secretion. Instead the RF B cells may play a role in the regulation of immune responses to antibody bound foreign and possibly self antigens. Such a physiologic role may be disrupted in RF associated autoimmune disease.

**FZ 434** CONSTRUCTION AND TOLEROGENICITY OF NOVEL IMMUNOGLOBULIN FUSION PROTEINS. Elias T. Zambidis and David W. Scott, Division of Immuology, University of Rochester Cancer Center, Rochester, NY 14642, U.S.A.

Isologous as well as heterologous immunoglobulins have been shown to be extremely effective as tolerogenic carriers for nearly thirty years. The efficacy of these proteins is presumed to stem from their long half-life *in vivo*, as well as their ability to crosslink surface IgM with Fc receptors. The concept of using IgG as a carrier molecule to induce unresponsiveness in the adult immune system has been exploited for simple haptens such as nucleosides as well as for peptides. In order to further evaluate the *in vivo* potential of these molecules in inducing tolerance to a defined peptide epitope, we have engineered a fusion protein of mouse $IgG_1$ and the immunodominant epitope 12-26 from bacteriophage lambda cI repressor protein. This 15-mer, which contains both a B-cell and T-cell epitope, has been engineered at the end of the N-terminus of a mouse H chain $IgG_1$ construct, thus creating a "clonable hapten-carrier" system. Another construct has been designed in which the 12-26 epitope is expressed in the third hypervariable region of the H chain. These H chain constructs have been electroporated into J558L cells and recombinant molecules have been purified from transfectoma supernatants and analyzed. The 12-26 epitope has been shown to be expressed in the primary as well as tertiary structure of the N-terminus 12-26-IgG1 by Western blots and ELISA using the mAb B3.11 which is specific for the 12-26 sequence. Competitive inhibition ELISA experiments show that this chimeric molecule effectively competes with synthetic 12-26 peptide for binding to the B3.11 mAb. *In vivo* and *in vitro* tolerance experiments with this chimeric immunoglobulin using a 12-26-flagellin fusion protein as immunogen are currently in progress. (Supported by USPHS Grant # A1-29691 and USPHS Grant #T32 GM-07356.)

*Structure/Function Studies of B Lymphocyte Receptors*

**FZ 435** DIFFERENTIAL EFFECTS OF THE RELATED MACROLIDES FK506 AND RAPAMYCIN ON HUMAN B LYMPHOCYTE ACTIVATION. K.M. Aagaard-Tillery, J.K. Braaten, and D.F. Jelinek, Dept. of Immunology, Mayo Clinic, Rochester, MN 55905

The immunosuppressive agent rapamycin (RAP), shows marked structural similarity to the potent macrolide lactone, FK506. Both agents have been reported to inhibit activation of mitogen-stimulated T and mixed T and B cell cultures, yet RAP appears to exert its inhibitory effects in a manner distinct from that of FK506. FK506 and RAP are therefore potent immunosuppressants with remarkable specificity, making both agents important tools for probing signal transduction events. The goal of this study was to use FK506 and RAP as tools to begin to dissect the activation pathways utilized by human B cells. The effects of both drugs on responses of highly purified B cells obtained from peripheral blood were assessed after stimulation with the polyclonal B cell activator *Staphylococcus aureus* (SA) in the presence or absence of interleukin 2 (IL2), or with phorbol ester (PMA) and the calcium ionophore, ionomycin (iono). RAP significantly inhibited SA driven B cell proliferation, whereas FK506 was without effect. Moreover, SA-activated B cells cultured with IL2 were similarly inhibited, and inhibition was observed after both 3 and 5 days of culture, suggesting that RAP does not function by delaying responses. By contrast, B cell proliferation after stimulation with PMA+iono was relatively insensitive to RAP treatment, yet was markedly inhibited by the addition of FK506. The effects of both drugs on B cell differentiation into Ig secreting cells was also examined. SA+IL2 stimulated B cells secreted significant amounts of IgM, IgG, and IgA, and the secretion of all three isotypes was completely inhibited by the presence of RAP, while FK506 was without effect. The ability of RAP to inhibit Ig secretion and B cell DNA synthesis when added as late as 48 hours prior to culture harvest suggests that RAP inhibits by blocking an event(s) that occurs in an ongoing and cyclic fashion, and not a one-time event required for initial activation. In an effort to determine more precisely the point of action of RAP in both IL2 dependent and independent responses, studies were therefore carried out to examine the effects of RAP on cell cycle progression, using the metachromatic dye, acridine orange. Results from these studies indicated that RAP inhibited event(s) prior to the $G_1$ to S transition point critical for both cell cycle progression, as well as cellular differentiation. In conclusion, our studies indicate that RAP and FK506 exert different effects on human B lymphocyte activation, and that RAP is inhibiting some event(s) necessary for continued cell cycle progression.

FZ 436 ANTI-IGM-MEDIATED TYROSINE PHOSPHORYLATION OF PI3 KINASE IS NOT ESSENTIAL FOR GROWTH INHIBITION OF A HUMAN B-LYMPHOMA CELL LINE, Margaret Beckwith[1], Robert G. Fenton[2], Ildy M. Katona[3], and Dan L. Longo[2]. [1]PRI/DynCorp, Inc., Biological Carcinogenesis and Development Program, [2]Biological Response Modifiers Program, NCI-FCRDC, Frederick, MD 21702 and [3]Departments of Pediatrics and Medicine, USUHS, Bethesda, MD 20814.

Crosslinking of membrane-bound IgM on an $IgM^+$, $IgD^+$ human B lymphoma cell line (RL) results in inhibition of cell proliferation. This receptor-mediated signalling involves phosphorylation of multiple proteins on tyrosine. The effect of anti-IgM on tyrosine phosphorylation and cell growth are augmented in the presence of the phosphatase inhibitor orthovanadate, and reversed in the presence of tyrosine kinase inhibitors. We wished to identify the major substrates of tyrosine phosphorylation, and determine their role in signalling through surface IgM and IgD. We show here that anti-IgM linked to acrylamide beads results in the phosphorylation of both the p85 and p110 subunits of phosphatidylinositol-3-kinase (PI3K). In contrast, we have not been able to demonstrate anti-IgM-induced phosphorylation of phospholipase C-γ1 (PLC-γ1) or GTPase activating protein (GAP), both of which have been identified as substrates of anti-Ig-induced phosphorylation in normal B lymphocytes. Furthermore, we have examined patterns of tyrosine phosphorylation and cell growth following treatment with the soluble conjugates of anti-IgM and anti-IgD to high molecular weight dextran. Although both of these reagents result in tyrosine phosphorylation and inhibition of RL cell growth, PI3K does not appear to be a major substrate. These data demonstrate that triggering of either IgM or IgD using a multivalent reagent can deliver growth inhibitory signals. In addition, there are clear differences in phosphorylation of PI3K following treatment with soluble vs. solid-phase anti-IgM, suggesting that phosphorylation of this molecule may not be directly involved in anti-Ig-induced growth inhibition.

FZ 437 INDUCTION OF GERMLINE TRANSCRIPT EXPRESSION BY IL-4: THE ROLE OF PROTEIN KINASE C AND $Ca^{++}$-DEPENDENT SIGNALING EVENTS, Michael T. Berton, Department of Microbiology, University of Texas Health Science Center, San Antonio, TX 78284

An important biological property of IL-4 is its ability to induce isotype switching to IgG1 and IgE in murine B cells *via* transcriptional activation of the unrearranged Cγ1 and Cε genes prior to switch recombination. Very little is known about the signaling events involved in this induction or about IL-4 receptor-mediated signaling in general. In this study, pharmacologic inhibitors and activators were employed to identify signal transduction pathways involved in the induction of germline γ1 transcript expression by IL-4 in an IL-4-responsive subclone of the B cell lymphoma line $BCL_1$ and in normal murine B cells. IL-4-mediated expression of the unrearranged Cγ1 gene was significantly inhibited (>90%) by H7, a potent inhibitor of protein kinase C, but not by HA1004, an inhibitor of cyclic nucleotide-dependent kinases. Direct activation of PKC in the absence of IL-4 by PMA, or the nonphorbol PKC agonists (-)-indolactam and mezerein, was not sufficient to induce germline γ1 transcript expression. However, costimulation of B cells with IL-4 and PKC agonists resulted in a significant enhancement of expression relative to that observed in B cells stimulated with IL-4 alone. There was no enhancement observed in response to IL-4 and 4αPDD, a phorbol ester that does not activate PKC. To determine whether $Ca^{++}$-dependent signaling events might also be involved in the induction of germline γ1 transcription, normal B cells were stimulated with IL-4 in the presence of cyclosporin A (CsA). CsA inhibited the expression of germline γ1 transcripts in a dose-dependent fashion in response to IL-4 alone, LPS and IL-4 or Th2 cells and specific antigen. Interestingly, CsA had no effect on the induction of germline γ1 transcripts in the inducible subclone of $BCL_1$. These studies suggest that at least certain IL-4-mediated responses involve both PKC activation and $Ca^{++}$-dependent signaling event(s). The role of these signaling events in the induction of germline ε transcription and their impact on the *trans*-acting factors involved in regulating germline γ1 and ε transcription are currently under investigation.

FZ 438 THE INTERACTION OF IG AND CLASS II MHC MEDIATED SIGNALING IN B CELL LINES AND SUBSETS OF B CELLS FROM NORMAL AND AUTOIMMUNE MICE. Gail A. Bishop*◇∞, Thomas J. Waldschmidt△ and Gary A. Koretzky◇, Depts. of Microbiology*Pathology△ and Internal Medicine◇, The University of Iowa, and the Iowa City VAMC∞, Iowa City, IA 52242.

Both normal splenic mouse B cells and the mouse B cell clone, CH12.LX, receive activation signals via their membrane MHC class II molecules. Splenic B cells from normal mice did not respond to a mixture of a non-mitogenic anti-IgM mAb (Bet-2) plus IL-4, but the addition of anti-class II mAb to these cultures induced proliferation. Similarly, CH12.LX cells, which endogenously produce IL-4 and require it for their continuous growth, secrete IgM when cultured with antigen or anti-Ig plus anti-class II mAbs. Splenic B cells were sorted by flow cytometry into $CD23^+$ and $CD23^-$ subsets. Both subsets responded to the combination of Bet-2, IL-4 and anti-class II, but the $CD23^-$ B cells showed a much higher proliferative response than the $CD23^+$ B cells. B cells purified from the spleens of young, asyptomatic mice of the autoimmune BWF1 strain responded to culture with Bet-2 with a marked *decrease* in proliferation. However, culture with IL-4 returned their proliferation to baseline levels, and addition of anti-class II mAbs completely restored the induced increase in proliferation seen in normal B cells. Again, these effects were greater in $CD23^-$ B cells. Similar findings were observed using the $CD23^-$ B cell clone CHB3. The growth of this clone is markedly inhibited by anti-IgM antibodies. This growth inhibition can be reversed, however, by the addition of anti-class II mAbs, although the latter have no effect themselves on B cell growth. Taken together, our findings using both B cell clones and freshly isolated B cells suggest that class II MHC molecules may receive signals which serve to rescue B cells from tolerogenic signals delivered through their membrane Ig molecules, and that B cells of the $CD23^-$ subset, which share many characterisitics with $CD5^+$ or "B1" B cells, are particularly responsive to these signals.

FZ 439 **Selective interactions between human B cell receptor subunits and cytoplasmic components.** Gaby Brouns, Evert de Vries, David Y. Mason* and Jannie Borst, Div. of Immunology, The Netherlands Cancer Institute, Amsterdam, The Netherlands and *Dept. of Haematology, John Radcliffe Hospital, Oxford, U.K.

The membrane bound form of Ig plays an important role in B cell development. At the pre-B cell stage the μ-protein associated with a pseudo light chain is believed to be functional in signal transduction, whereas μ-chain associated with a conventional light chain plays this role on mature B cells. We have compared the pre B cell receptor (BCR) and the mature BCR structures in terms of signaling competence.

It was demonstrated that, like the mature BCR, the pre-BCR is associated with a heterodimer consisting of mb-1 and B29 proteins. At this developmental stage the receptor is associated with the *src*-like kinases *lyn*/*fyn* and *lck*, which has also been described for the mature BCR complex. Qualitative differences in participation of *src*-like kinases have been observed between pre-BCR and BCR. In addition serine/threonine kinase activity was observed in both receptor complexes. In pre-BCR and BCR, mb-1, B29 and the *src*-kinases are substrates for tyrosine as well as serine/threonine phosphorylation *in vitro*. Both complexes contain a 43 kD phosphoprotein (p43) which is *in vitro* phosphorylated only on serine/threonine residues. Substrate specificities of the associated kinases have not yet been determined.

Using differential detergent extractions followed by re-precipitation, we have investigated the direct physical interactions between the various components of the BCR complex (μ-chain versus mb-1/B29) and the kinases mentioned above. From these studies we conclude the following: 1) the mb-1/B29 heterodimer interacts strongly with *lyn* and *fyn*. 2) No direct interaction between *lck* and components of the BCR is observed, which leaves the possibility that this kinase is brought into the complex via an accessory molecule. 3) The heavy chain is associated with phosphoproteins of 43, 57 and 72 kD.

**FZ 440** sIg mediated desensitization of PLC dependent pathways occurs at a point distal to tyrosine kinase (TK) activation. M. Brunswick*, C. June#, A. Burkhardt0, J. Bolen0, J. Mond+, *FDA Bethesda MD, #NMRI Bethesda MD, 0Bristol Myers Squibb Princeton NJ, +USUHS Bethesda MD.

Cross linking of the B cell membrane Ig receptors leads to a rapid increases in $[Ca^{2+}]_i$ followed by a prolonged period of desensitization during which subsequent cross linking of either the homologous or heterologous sIg no longer stimulates additional increases in $[Ca^{2+}]_i$. In view of the fact that sIg mediated TK activation is required for subsequent activation of PLC leading to increases in $[Ca^{2+}]_i$, we wished to determine whether desensitization is mediated at the early stage of TK activation or at a later point. Resting mature B cells were incubated for 60 minutes in either medium or anti-Ig, followed by extensive washing and readdition of anti-IgD or addition of anti-IgM. Control cells showed normal increases in tyrosine phosphorylation (TyrP) and $[Ca^{2+}]_i$ following exposure to anti-IgD or anti-IgM. Cells that were incubated with anti-IgD showed no further increases in TyrP or in $[Ca^{2+}]_i$ after addition of anti-IgD, reflecting the fact that IgD was modulated from the cell surface. Most interestingly however, the same cells when exposed to anti-IgM showed no increase in $[Ca^{2+}]_i$ reflecting the fact that they were desensitized but they showed increases in TyrP of a comparable number of substrates as was seen in control cells. The data suggest that the absence of additional increases in $[Ca^{2+}]_i$ in desensitized cells does not reflect the suppression of TK activation but rather reflects suppression of a distal step.

**FZ 441** EXPRESSION OF p56*lck* IN THE J558LμM3 CELL LINE PARTIALLY RESTORES MEMBRANE IMMUNOGLOBULIN-MEDIATED SIGNAL TRANSDUCTION, Mary-Ann Campbell & Bartholomew M. Sefton, MBVL, The Salk Institute, 10010 N Torrey Pines Rd La Jolla CA 92037

Crosslinking of the B lymphocyte antigen receptor, membrane immunoglobulin (Ig), with anti-Ig antibodies stimulates the rapid protein tyrosine phosphorylation of a number of substrates including Igα, Igβ, phospholipase Cγ, MAP-2 kinase, $p95^{vav}$ and CD22. B lymphocytes express a number of protein tyrosine kinases of the *src*-family including *lyn*, *fyn*, *blk* and *lck*, all of which have been shown to physically associate with membrane Ig.

The membrane IgM-positive, murine myeloma cell line, J558LμM3 is unable to respond to crosslinking of membrane Ig as measured by changes in calcium flux but this response is restored if the cells are transfected with the protein tyrosine phosphatase CD45. CD45 has been shown to activate *lck* kinase by dephosphorylation of a negative regulatory site, tyrosine 505. We have found that J558LμM3 cells do not express detectable levels of *lck*, *fyn* or *lyn* kinase activity and their membrane IgM is not associated with significant tyrosine kinase activity. Crosslinking of membrane IgM on these cells has no detectable effect on the levels of protein tyrosine phosphorylation.

To determine if expression of an activated tyrosine kinase is sufficient to promote signal transduction in J558LμM3 cells we have transfected these cells with wild-type *lck* kinase (*lck*-wt) and an activated form of *lck* kinase where the negative regulatory site, tyrosine 505, has been mutated to phenylalanine (*lck*-F505). We have found that expression of *lck*-F505 leads to an increase in the level of constitutive protein tyrosine phosphorylation. Crosslinking of membrane IgM results in an increase in the tyrosine phosphorylation of an 80kD molecule in cells expressing *lck*-F505 and to a lesser extent in those expressing *lck*-wt. However we have found that crosslinking of membrane IgM on these cells does not stimulate an increase in cytoplasmic $Ca^{2+}$ concentration. Thus expression of an activated protein tyrosine kinase in J558LμM3 cells is only partially able to restore transmembrane signalling in response to crosslinking of membrane Ig.

**FZ 442** MOLECULAR AND CELLULAR CHARACTERIZATION OF EGR-1 FUNCTION IN B CELL ACTIVATION AND TOLERANCE, Julie A. Carman and John G. Monroe, University of Pennsylvania, Philadelphia, PA 19104

The immediate early gene, egr-1, is rapidly and transiently induced following ligation of the B cell antigen receptor. Induction of egr-1 expression is correlated with, and required for a positive B cell activation response. Antisense egr-1 blocks anti-IgM-induced activation of mature splenic B cells. We have found that immature stage B cells from adult mouse bone marrow do not express egr-1 in response to antigen receptor crosslinking. These B cells are also not activated by this stimulus, suggesting a cause and effect relationship between lack of egr-1 inducibility and the unresponsiveness of these cells. Egr-1 encodes an 80 kD nuclear glycoprotein with transcriptional regulatory activity. It may therefore serve as a critical link between early biochemical signalling events and changes in gene expression required for long term cellular responses. We are using two approaches to further define the role of egr-1 in the development of positive B cell activation responses. A transient co-transfection system was developed to define the regions of the egr-1 protein important for biological activity. To define a role for egr-1 in the regulation of tolerogenic and activation responses by the B cell during development, we have constructed a transgenic mouse line that expresses egr-1 under the control of the immunoglobulin heavy chain enhancer. In this line, constitutive egr-1 expression will be maintained in immature bone marrow-derived B cells. The effects of this expression on B cell development and tolerance are to be examined. Recent progress in these two systems will be presented.

**FZ 443** G-PROTEIN EXPRESSION DURING B LYMPHOCYTE MATURATION, PROLIFERATION AND DIFFERENTIATION.

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The molecular mechanisms underlying the development of haemopoietic pluripotential stem cells into antigen-specific, antibody-secreting plasma cells and memory cells are as yet poorly understood. However, I have recently shown that at least two events in B cell activation and proliferation are regulated by G-proteins [1,2]. The G-proteins comprise a large and structurally diverse superfamily of proteins, which bind and hydrolyse GTP and are involved in the regulation of key cellular events such as transmembrane signalling, intracellular trafficking, proliferation and differentiation [3]. Haemopoietic lineage- and differentiation-restricted G-proteins have recently been identified [4]. Thus, it is planned to target critical regulatory events in B cell ontogeny by probing for novel sites of G-protein regulation of B cell maturation and differentiation. In particular, the correlation between G-protein expression and B cell maturation will be investigated. Thus, data will be presented demonstrating the profiles of G-protein expression in B cells (and other haemopoietic lineages) at different stages of maturation. G-protein expression will be determined by Western Blot analysis (using specific anti-G-protein antibodies) of haemopoietic cells and cell lines frozen at various states of maturation. This approach will be supported by data from 3-colour FACS analysis of permeabilised cells simultaneously stained for lineage/differentiation-specific cell surface antigens and intracellular G-proteins.

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**FZ 444 THE B-CELL ANTIGEN RECEPTOR-ASSOCIATED PROTEINS Ig-α AND Ig-β HAVE DIFFERENTIAL SIGNAL TRANSDUCTION CAPACITIES.** Grace Ku[1], J. C. Cambier[2] and Bernard Malissen[1]
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The B-cell antigen receptor (BCR) complex is an oligomeric structure composed of membrane-bound immunoglobulin (Ig), noncovalently associated with dimers of disulfide-linked Ig-α and Ig-β polypeptides. The Ig-α and Ig-β proteins, which are products of the mb-1 and B29 genes respectively, are thought to couple antigen recognition by BCR to intracellular signal transduction events. The cytoplasmic domains of both Ig-α and Ig-β contain the antigen-receptor consensus sequence motif $D/EX_7D/EX_2YX_2L/IX_7YX_2L/I$, originally identified by Reth (Reth, 1989 Nature, **338**:383-384). Recent studies with T-cell receptor/CD3-ε and -ζ have shown that this antigen-receptor sequence motif can function independently as a signal transducer for T-cell activation (Letourneur and Klausner, 1992 Science, **255**:79-82 , Wegener et al., 1992 Cell, **68**:83-95). To determine whether these antigen receptor consensus sequences in Ig-α and Ig-β can function analogously in B and T lymphocytes, chimeric CD8α/Ig-α or CD8α/Ig-β fusion proteins were constructed and were stably expressed in B and T cell lines. Upon stimulation with an anti-CD8α monoclonal antibody, Ig-α alone is sufficient for mediating and sustaining both early (eg. tyrosine kinase activity) and late (eg. IL-2 production) events associated with activation by an intact BCR/TCR. However, results from parallel studies with Ig-β demonstrate that this polypeptide is inert for transducing signals which lead to tyrosine phosphorylation as well as IL-2 synthesis. The ability of the Ig-α and Ig-β fusion proteins to mobilize intracellular $Ca^{2+}$ in B and T cells is currently being assessed. These data suggest that Ig-α and Ig-β may function in distinct second messenger pathways.

**FZ 445** THE CD19 COMPLEX ON HUMAN B CELLS CONTAINS THE SRC-FAMILY KINASE LYN AND HAS CHARACTERISTICS OF A SIGNALING MODULE, Arjan C. Lankester, Carel J.M. van Noesel, Gijs M.W. van Schijndel and René A.W. van Lier, Dept. of Clin. Viro-Immunology, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and Laboratory of Experimental and Clinical Immunology of the University of Amsterdam, Amsterdam, The Netherlands

The 95 kDa CD19 transmembrane glycoprotein is a member of the Ig-superfamily, which is expressed during all stages of human B cell differentiation. At the surface of mature human B cells the CD19 complex can be found non-covalently associated with complement receptor type 2 (CR2/CD21). Upon their cross-linking both CD19 and CR2 have been shown to generate transmembrane signals which are agonistic for B cell activation, although the mechanism remains unknown. Since protein phosphorylation is regarded as an early and important event in the transduction of signals following surface receptor ligation, we have investigated whether the CD19 complex contains protein kinase activity and/or whether CD19 serves as a substrate for such activity itself. Biochemical analysis of CD19 immunoprecipitates, using both *in vitro* and *in vivo* phosphorylation assays, revealed that CD19 is phosphorylated, mainly on serine residues. In addition, two other molecules of 53 and 56 kDa, which were co-precipitated, appeared to be phosphorylated also on tyrosine residues. Using specific antisera, they were identified as the two splice forms of the src-family member *lyn*. The cytoplasmic tail of the CD19 molecule shows some interesting features. Besides a number of conserved serine and threonine residues, nine conserved tyrosine residues, encoded by seven exons, are present, which are flanked at several positions by the same amino acids. Comparison with the human mb-1 and b-29 cytoplasmic tails revealed limited similarity.
Both CD19 and CR2 are known to physically associate with the B cell antigen receptor, following its ligation. Therefore, the recognition of foreign peptide, opsonized with complement fragments, might result in co-ligation of these receptor complexes. Consequently, the CD19 associated *lyn* molecules are brought in vicinity of potential substrates, whereas the tyrosines in the CD19 tail, when phosphorylated, could serve as binding sites for second messengers.

**FZ 446** CD22 IS PART OF THE HUMAN sIgM/B CELL ANTIGEN RECEPTOR SIGNALING COMPLEX
Corinne Leprince#, Kevin A. Draves#, J. A. Ledbetter‡, Robert A. Geahlen* and Edward A. Clark#. #Dept of Microbiology, SC42, University of Washington, Seattle WA98195, ‡Bristol-Myers Squibb Research Institute, Seattle and *Purdue University, West Lafayette, IN 47907.

CD22 is a B cell restricted marker expressed in a pattern similar to the sIg/B cell antigen receptor. Cloning of CD22 cDNAs revealed that it is a member of the Ig superfamily and an adhesion molecule which can interact with its CD45RO ligand on T cells. CD22 mAb potentiate subsequent B cell response to anti-IgM Ab suggesting a functional relationship between CD22 and sIgM.

In the present work, we analyzed in more detail how sIgM and CD22 signaling pathways are related. After digitonin cell lysis, CD22 was immunoprecipitated in association with a protein kinase inducing the tyrosine phosphorylation of CD22 as detected by an *in vitro* assay. Phosphorylated CD22 migrated in SDS-PAGE at the same position as a previously described 150 kDa component of the sIgM complex (Eur. J. Immunol. 22:2093, 1992). We found that this 150 kDa polypeptide is indeed CD22. First, CD22 could be removed from the sIgM complex by sequential immunoprecipitations. Second, a rabbit antiserum specific for a synthetic peptide of CD22 reacted in Western blotting with the 150 kDa protein present in sIgM immunoprecipitates. Moreover, a cross-talk exists between CD22 and sIgM: stimulation of viable B cells via sIgM induced a rapid and dramatic tyrosine phosphorylation of CD22. In addition to CD22, we also identified a 75 kDa component in the human IgM complex to be spleen tyrosine kinase, SYK. We propose that CD22 is a crucial signal transducing molecule which can enter the sIgM complex and potentially interact with MB1/Igα and B29/Igβ and protein kinases such as SYK. The analysis of the intracytoplasmic aminoacid sequence of CD22 revealed that it contains 6 conserved tyrosines, 5 of which are organized in a AHRI-like motif in an arrangement strikingly similar to that of the ζ chain of the CD3/TCR complex, which has been shown by Weiss and coworkers to bind to a T cell homolog of B cell SYK.
(Supported by NIH grants GM37905 and GM42508 and by a Fogarty International Fellowship.)

**FZ 447** IDENTIFICATION OF B CELL PHOSPHOPROTEINS THAT BIND TO THE SH2 DOMAIN OF BLK, Sami Malek and Stephen Desiderio, Department of Molecular Biology and Genetics and Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

As part of an effort to elucidate the function of the B cell-specific protein-tyrosine kinase Blk, a member of the Src family, we have identified proteins that bind the Src-homology region 2 (SH2) domain of Blk. The SH2 domains of murine Blk, Lyn, Fyn and c-Abl and the N-terminal SH2 domain of murine GAP were expressed as fusions to glutathione-S-transferase (GST), purified, and immobilized by binding to glutathione-Sepharose. The resulting affinity matrices were used to adsorb proteins labeled in vivo with $^{32}P$ from extracts of the mature B cell line A20. We observed no binding to an affinity matrix containing unfused GST. The Blk SH2 fusion protein bound 12 - 15 distinct phosphoproteins, most of which reacted with the 4G10 anti-P-Tyr antibody. The P-Tyr content of these proteins, as assessed by reactivity to the 4G10 antibody, was greatly increased if sIgG was crosslinked before A20 extracts were made. An R145K substitution in the FLVRESE motif of Blk SH2 abolished binding to all of these proteins, as did four different Blk SH2 deletion mutations; an S147C substitution reduced binding about 5-fold. Binding was greatly reduced by inclusion of 40 mM P-Tyr, but unaffected by similar concentrations of P-Ser or P-Thr.

Three prominent phosphoproteins of 72-74 kD, 115 kD and 130 kD bound to the Blk, Lyn, Fyn and GAP SH2 domains but not to the c-Abl SH2 domain, to which no specific binding was detected under these conditions. Phosphoaminoacid analysis of these three proteins revealed that each contained P-Ser, P-Thr and P-Tyr. For the Blk, Lyn, Fyn and GAP SH2 domains, several selective interactions were observed. A 95 kD phosphoprotein was bound only by the Blk and GAP SH2 domains, and a 90 kD phosphoprotein was bound by the Blk, Lyn and GAP SH2 domains but not by that of Fyn. Two SH2-binding proteins were recovered from A20 but not from the T cell line EL-4: the prominent 72-74 kD protein described above and a less abundant 60 kD protein. The Blk, Lyn, Fyn and GAP SH2 domains each bind at least one tyrosine kinase activity and one serine/threonine kinase activity, and of the predominant tyrosine kinase substrates observed in these eluates, one is specifically recovered from the Blk SH2 matrix.

**FZ 448** MUTATIONS IN MEMBRANE IMMUNOGLOBULIN M TRANSMEMBRANAL DOMAIN ALTER BINDING OF Igα AND Igß. Carolyn H. Michnoff, Vandana S. Parikh, David L. Lelsz and Philip W. Tucker, Department of Microbiology, UT Southwestern Medical Center, Dallas, Texas 75235

Binding of Igα (mb-1 gene product) and Igß (B29 gene product) were compared between wild type membrane immunoglobulin M (mIgM) and mutant mIgM from stable transfectants of the murine B cell lymphoma M12.4 ($mIgM^-$, $mIgD^-$). Digitonin lysates of cells were immunoprecipitated with anti-mouse μ heavy chain and protein G agarose. Immunoprecipitates were separated by nonreducing and reducing, respectively, two-dimensional SDS polyacrylamide gel electrophoresis. Antibodies against peptides of Igα and Igß were used in western blots. Substitution of mIgM spacer, transmembrane and cytoplasmic domains (EGEVNAEEE GFE/NLWTTASTFIVLFLLSLFYSTTVTLF/KVK) with that of I-Aα (EPEIPAPMSELTE/TVVCALGLSVGLVGIVVGTIFIIQGL/RSGG GTSRHPGPL) prevented association of Igα and Igß with that of the mutated mIgM. In preliminary studies, Igα and Igß did not bind to mutant mIgM containing I-Aα sequences in the first third of the μ transmembrane domain (EGEVNAEEEGFE/TVVCALGLFIVL FLLSLFYSTTVTLF/KVK). However, mutant mIgM containing $mIgG_2B$ spacer, transmembrane and cytoplasmic domains (GLDLDDICAEAKDGELD/GLWTTITI FISLFLLSVCYSASVTLF/KVKWIFSSVVELKQKISPDY) was able to bind Igα and Igß. Antigen or anti-μ $(Fab')_2$ antibody crosslinking of the chimeric $mIgM\text{-}mIgG_2B$ stimulated intracellular $Ca^{2+}$ mobilization. These results suggest critical binding determinants for Igα and Igß reside within the $NH_2$-terminal 8 residues of mIgM transmembrane. Moreover, substitution of $mIgG_2B$ spacer, transmembranal and cytoplasmic domains for that in mIgM are sufficient for receptor binding of Igα and Igß and the initial signal transduction response of intracellular $Ca^{2+}$ mobilization.

**FZ 449** ROLE OF *SRC* FAMILY PROTEIN TYROSINE KINASES (PTK) IN B-CELL SIGNALING AND TOLERANCE. David W. Scott, Arti Gaur, and Xiao-rui Yao. Division of Immunology, University of Rochester Cancer Center, Rochester, NY, USA, 14642.

Protein tyrosine phosphorylation events have been implicated in signal transduction in a number of cell types. To investigate the role of the *src* PTKs in B-cell signaling and tolerance, we have examined normal mouse spleen cells and a series of B-cell lymphomas for a) the presence and activity of *src*-encoded kinases; b) the effect of PTK inhibitors on signal transduction; c) the ability of antisense oligos to influence functional responsiveness. In normal B cells, blk, lyn, and fyn kinases were all present and PTK inhibitors (tyrphostin, genistein, herbimycin) inhibited signaling to different extents in anti-phosphotyrosine western blotting experiments. When splenic B-cells were treated with tyrphostin and washed, they were able to produce antibody in response to antigen, but were partially inhibited in tolerance induction through surface IgM. In contrast, herbimycin-treated spleen cells were blocked in membrane Ig-mediated signaling while retaining LPS responsiveness for proliferation and antibody synthesis; interestingly, blk protein was preferentially depleted in herbimycin-treated splenic B cells. Studies with antisense oligos for *blk* in CH31 lymphoma cells indicate that the blk kinase may play a critical role in negative signaling of this lymphoma line. Since several non-growth inhibitable cells possessed both blk and fyn, we hypothesized that the fyn PTK might modulate negative signaling. Infection of growth-inhibitable B-cell lines with a retroviral vector containing *fyn* led to expression of this kinase. However, the presence of *fyn* alone did not reverse the growth inhibitory phenotype in a number of clones, a result suggesting that the presence of this PTK *per se* is not sufficient to resistance to anti-μ. Our studies begin to address the differential roles and duplication of function of the *src* family of PTKs in B-cell signaling and tolerance. (Supported by USPHS grants, AI29691, CA55644, and ACS grant, IM-495C)

**FZ 450** TYROSINE PHOSPHORYLATION OF PHOSPHOLIPASES Cγ1 AND γ2 AFTER sIgM STIMULATION OF B LYMPHOCYTES IS DEVELOPMENTALLY REGULATED, Amy L. Sillman, Amy Yellen-Shaw, Mark Coggeshall, and John G. Monroe, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104

The signalling cascade initiated by stimulation of sIgM on B lymphocytes is developmentally regulated. While mature (sIgM+, sIgD+) B lymphocytes enter cell cycle after stimulation through sIgM, immature B lymphocytes do not. We have previously shown that the difference in signalling between immature and mature B lymphocytes differs depending upon the anatomic site of origin. In immature B lymphocytes from the neonatal spleen, there is a difference in transmembrane signalling that lies upstream of inositol phospholipid (PI) hydrolysis. We have begun studies to define the molecular basis for this uncoupling of sIgM from PI hydrolysis in neonatal splenic immature B lymphocytes. Recent studies have shown that regulation of phospholipase C-mediated PI hydrolysis involves tyrosine kinase activity. We have observed by anti-phosphotyrosine immunoblot analysis that tyrosine phosphorylation occurs in both mature and immature B lymphocytes after sIgM stimulation, with at least one notable difference. A band migrating at 150 kD becomes tyrosine phosphorylated in mature, but not immature B lymphocytes after sIgM stimulation. Anti-phospholipase C immunoblot analysis shows that this tyrosine-phosphorylated band co-migrates with phosphlipases Cγ1 and γ2. Immunoprecipitation of phospholipases Cγ1 and γ2 and subsequent anti-phosphotyrosine immunoblot analysis demonstrates that, although mature and immature B lymphocytes express equivalent amounts of phospholipases Cγ1 and γ2, these enzymes become tyrosine phosphorylated after sIgM stimulation only in mature B lymphocytes. We are currently investigating the relevant difference in tyrosine kinase activity that is involved in this developmentally-regulated difference in PLCγ phosphorylation following sIgM crosslinking.

**FZ 451** ROLE OF IMMUNOGLOBULIN RECEPTOR COMPLEX IN THE GROWTH REGULATION OF WEHI 231 CELLS, Yongjian Wu, Naoto Iwabuchi, Hai P. Nguyen and Nobumichi Hozumi, Departments of Immunology and Molecular Genetics, University of Toronto and Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada M5G 1X5

The growth of murine B lymphoma line WEHI 231 is inhibited by surface IgM crosslinking, making it an excellent model to study B cell tolerance. We have previously transfected IgD and other chimeric Ig's into WEHI 231 and assayed for the ability of these molecules to deliver a negative signal. Our results indicated that membrane region of the surface IgM is not sufficient, and other extracellular domain(s) are required for the induction of growth inhibition. This finding is consistent with the discovery that Ig may signal through associated molecules (Igα and β) that interact with Ig domains on both sides of the membrane. Here, we show that Igα is associated with endogenous and various transfected Ig's. Further, we provide evidence for its essential role in mediating the downstream biochemical events. One of the early events is the activation of protein tyrosine kinases and consequent phosphorylation of other substrates on tyrosine residues. We demonstrate the ability of these molecules to couple receptor crosslinking to this pathway and show that *lyn*, a *src* family tyrosine kinase, is constitutively associated with the Ig receptors. To further support a role for tyrosine kinases, we obtained stable *fyn* (another *src* family tyrosine kinase) transfectants of WEHI 231 cells which do not express endogenous *fyn*. As the result of exogenous *fyn* expression, their sensitivity to IgM-mediated killing is enhanced. We are currently investigating the mechanisms by which the crosslinking of surface Ig induces the tyrosine kinase activation and ultimately B cell activation/tolerance. Supported by NCIC.

**FZ 452 ROLE OF CD45 IN IgM RECEPTOR-MEDIATED B CELL SIGNALING.** Hidetaka Yakura, Mami Ogimoto, Tatsuo Katagiri, Kiminori Hasegawa, and Kazuya Mizuno. Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo 183, Japan

Cross-linking of membrane IgM receptor on B cells by anti-IgM antibody leads to protein tyrosine phosphorylation and phospholipase C-catalyzed breakdown of phosphatidylinositol bisphosphate, which results in the activation of protein kinase C and the release of calcium from cytoplasmic stores. These biochemical changes result in activation in mature B cells but in immature B cells, induce growth arrest and apoptosis presumably as a result of endogenous endonuclease gene activation. To study the regulatory role of membrane-bound protein tyrosine phosphatase (PTP) CD45 in this process, CD45-negative clones were isolated from immature B cell line WEHI-231 by treatment with a mutagen MNNG, complement-mediated cytotoxicity and subsequent limiting dilution. The isolated CD45-negative clones were then compared to the parental line with respect to anti-IgM antibody-induced biochemical changes. Anti-IgM antibody induced tyrosine phosphorylation of about 10 proteins in WEHI-231 cells, but in CD45-negative clones, tyrosine phosphorylation was not strongly induced, in particular 155 kd, 123 kd, 72 kd, 68 kd, 61-65 kd and 56 kd molecular species. These results suggest that initial signaling through IgM receptor is tightly controlled by CD45 and that the regulatory site of CD45 may be proximal to that of other tyrosine kinases associated with IgM receptor complex. Further, the hypophosphorylated molecules could be candidates for the substrate of CD45 PTP activity. This possibility is currently under investigation. We next examined whether this initial alteration in tyrosine phosphorylation is in any way involved in signal transduction and gene activation. In the events, CD45-negative clones were more susceptible to anti-IgM antibody-induced growth arrest than the parental line and showed more prominent DNA fragmentation patterns, suggesting that IgM receptor-mediated B cell growth arrest and apoptosis is negatively regulated by CD45. Taken together, regulation of tyrosine phosphorylation by CD45 seems to be one of the key elements in IgM receptor-mediated signaling events.

*Late Abstracts*

THE κ-CHAIN ENHANCER CONFERS DEMETHYLATION UPON THE Jκ REGION IN MATURE B-CELLS IN A **POSITION DEPENDENT** MANNER ONLY

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We are studying the mechanism and role of DNA methylation in the regulation of Ig κ-chain gene expression during B-cell development. We methylated the κ-chain gene in vitro using bacterial methylases and introduced the DNA stably into non-lymphoid and B-lymphoid cell lines. The ability to actively demethylate an exogenous κ-chain construct appears to be not only lymphoid specific but also cell-stage specific, since demethylation did not occur in a pre- B-cell line , where the endogenous gene is inactive and methylated. The demethylation was sequence specific, and restricted to the gene region itself. Moreover, a methylated κ-chain gene deleted of its promoter undergoes specific demethylation in B-cells. Thus, transcription per se is not sufficient for the demethylation process. In contrast, a methylated enhancer deleted κ-chain gene stays methylated in B-cells. Therefore, the κ-chain enhancer plays a vital role in the demethylation event. But most interestingly, a fragment containing the κ enhancer can confer B cell-specific demethylation upon the Jκ region in an orientation independent but position **dependent** manner. Thus, the specificity of the demethylation resides within a fragment containing the transcriptional enhancer; similar to a transcriptional enhancer it can demethylate from a distance and in an orientation independent manner, but unlike a transcriptional enhancer it can exert its effect in one position only.

**SIMULTANEOUS PRESENCE, IN ONE SERUM, OF FOUR MONOCLONAL ANTIBODIES WHICH MIGHT CORRESPOND TO DIFFERENT STEPS IN THE CLONAL EVOLUTION FROM POLYREACTIVE TO MONOREACTIVE ANTIBODIES.** **Guillaume Dighiero[1], Monique Houdayer[1], Jean-Pierre Bouvet[1], Annie Wolff[2], Christian Magnac[1], Jean-Claude Guillemot[3], Luis Borche[1]. [1]Institut Pasteur, [2]Collège de France, Paris, [3]Sanofi Elf Bio Recherches, Labège.**

**Three monoclonal IgG of different subclasses (IgG1, IgG2 and IgG4) and one IgA1 were isolated from the serum of patient Per suffering from an immunocytic sarcoma. All four monoclonal immunoglobulins shared the same N-terminal sequence of their heavy chains (VH3). Furthermore, their κ chains exhibited identical isoelectric charges and N-terminal sequences (VK2), and expressed the same private idiotope. A strong antitubulin activity was found in IgA1Per and in two of the three monoclonal IgGPer. The specificity was restricted to tubulin for IgA1Per and IgG4Per, whereas IgG1Per also displayed significant polyreactive bindings and IgG2Per failed to react with any of the antigens tested. The monoreactive IgG4Per, as well as the polyreactive IgG1Per, bound a large peptide in the central part of both α and β subunits of tubulin (amino acid position 100 to 300). In contrast, the monoreactive IgA1Per bound to a rarely detected epitope close to residue 310 of these subunits. The tubulin epitope recognized by polyreactive IgG1Per was similar to that of germline-encoded polyreactive antibodies. It is hypothesized that IgG4Per and IgA1Per producing cells derive from the IgG1Per polyreactive clone after somatic events leading to the production of monoreactive antibodies.**

**DIFFERENTIAL USAGE OF DNA SUBSTRATES IN PRIMARY AND SECONDARY SWITCH RECOMBINATION,** A.L. Kenter, R. Wuerffel, G.V. Merkulov, and R. Shefner, Department of Microbiology and Immunology, University of Illinois College of Medicine, Chicago, IL 60612

The deletion looping out model for switch recombination predicts that the intervening DNA between switch (S) regions will be excised as a circle. The reciprocal circular deletion products of Ig switch recombination have been recovered from mitogen activated spleen cells. We reasoned that since switch recombination is clearly focused on S regions, switch specific DNA-binding proteins might facilitate the alignment of these regions prior to recombination. The Sγ3 specific DNA-binding proteins, SNAP and SNIP/NF-κB interact with two discrete regions of the Sγ3 tandem repeat, the A and B sites. Recombination breakpoints in the Sγ3 switch region were found to significantly correlate with the binding sites of the Sγ3 switch-binding proteins. We now report the conservation of the SNIP and SNAP binding sites in Sγ2b and Sγ1 DNA. SNIP/NF-κB interacts with its cognate sites in Sγ2b and Sγ1 DNA as determined by mobility shift assays, competition-binding studies and supershift analysis using an antiserum specific for the p50 component. SNAP was found to specifically bind to Sγ2b and Sγ1 by mobility shift assays and competition binding studies. SNAP is composed of two closely traveling mobilities which do not separate upon partial purification. SNAP A1 and A2 were found to be distiguishable by differential phosphorylation states. The sites at which recombination occurs in the Sγ2b and Sγ1 switch regions have been analyzed statistically and found to correlate with the SNIP/NF-κB and SNAP binding sites. Distinctions have been found regarding the usage of DNA substrates within the tandem repeat between primary (μ->γ) and succesive (γ->x) switch recombination.

EXPRESSION OF CD71 (TRANSFERRIN RECEPTOR) IN THE SUBPOPULATION OF C-KIT+ EARLY HAMATOPOIETIC PROGENITORS, Tatsuo Kina, Eiji Takayama, Katsuya Wada, Yoshimoto Katsura, Chest Disease Research Institute, Kyoto University, Kyoto 606, and Yumi Matsuzaki, Hiromitsu Nakauchi, Rikenn Institute, Tsukuba 305, Japan.

In normal hematopoiesis, proliferation of stem cells is strictly regulated by the hematopoietic microenvironment. To investigate the mechanisms of stem cell proliferation, it seems necessary to distinguish the dividing cells from non-dividing stem cells. CD71 (transferrin receptor) seems to be a marker for this purpose, since it is known to be expressed in the cycling cells. By using newly established mAb (HANA-5) against CD71, we investigated the expression and function of CD71 in the murine hematopoietic system. In the bone marrow, 20% of cells were stained with HANA-5. Although 90% of this population co-expressed an erythroid marker (TER-119), the remaining population (1-2% of total bone marrow cells) expressed no other lineage markers (Gr-1, Mac-1 and B220). A half of the Lin$^-$c-kit$^+$ population was also stained with the HANA-5 Ab, whereas no HANA-5 reactive cells was observed in the Lin$^-$Sca-1$^+$ stem cells. Addition of the HANA-5 Ab to the in vitro hematopoietic systems resulted in the complete inhibition of both lymphoid and myeloid development. Although the formation of CFU-Mix, BFU-E and CFU-E colonies were completely blocked by the HANA-5 Ab, both CFU-GM and CFU-IL7 colonies were not affected substantially. These results strongly suggest that CD71 is expressed in the early cycling cells within the c-kit$^+$ stem cell population and that this cycling population is likely to be the early progenitors of all hematopoietic lineages.

**ABSTRACT TEMPLATE AND INSTRUCTIONS SPLANCHNOPLEURA FROM 9-DAY-OLD MOUSE EMBRYOS CONTAINS B-CELL PROGENITORS.** **Miguel A.R. Marcos, Isabelle Godin, Maria L. Gaspar, Antonio Coutinho, and Françoise Dieterlen-L., Universidad de Salamanca, Spain, Institut Pasteur and Institut d'Embryologie, Nogent-sur-Marne, Paris, France.**

The potentialities of hematopoietic stem cells (HSC) might change with developmental time and/or origin while their expression might depend on the microenvironment in which they evolve. To test the potentialities of HSC present in mouse embryonic areas (8-9,5 day old BALB/c embryos, 5-20 somites), we undertook grafting experiments under the kidney capsule of CB.17-SCID recipients. When analyzed 2-5 months post-grafting, recipients of either yolk sac or very early embryos (<7somites) did not reveal lymphoid repopulation. SCID mice grafted with paraaortic splanchnopleura (9-days embryos; 10-18 somites) showed, however, a normal population of B-1a (IgM$^{a+++}$B220$^{dull}$CD5+) cells in coelomic cavities (IgM$^a$ donor allotype). Spleens from these recipients had only a minor population of surface IgM+ B cells, but normal numbers of IgM$^a$-secreting plasma cells, as compared to adult BALB/c mice. Their bone marrows were devoid of mature B lymphocytes and plasma cells, and were unable to retransfer donor type, B cells to secondary recipients. It seems that, concerning lymphoid development, the actual fate of very early HSC located in paraaortic splanchnopleura is restricted to the generation of B-1a lymphocytes. Whether this is a feature intrinsic to these progenitors, or depends on regulatory interactions inside the graft microenvironment requires further analysis. Cellular and molecular characterization of the embryonic HSCs is being underway.

AN IGG WITHOUT HINGE ACTIVATES COMPLEMENT

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The hinge region of antibodies serves as a "connecting structure" with cysteines making disulfide bonds between heavy chains. We have engineered $IgG_3$ by deleting the hinge exons from the $\gamma 3$ heavy chain gene. The hingeless $IgG_3$ has no covalent bonds between its heavy chains, and is found to be inactive in mediating complement lysis (CML). The hinge is also believed to serve a spacer function, separating Fab from Fc.
The ability of C1q (the first component of the complement cascade) to interact with its binding site on CH2 presupposes that the Fabs do not cover these determinants. We have modified the hingeless $IgG_3$ mutant by introducing a cysteine between Ala-231 and Pro-232, the first and second amino acid in CH2, respectively. The resulting mutant $IgG_3$ molecules make disulfide bonds between heavy chains, and are more active in CML than $IgG_3$ wild type. We conclude that covalent linking of heavy chains between Fab and Fc is a prerequisite for CML, whereas spacing of Fab and Fc is not.
It is reasonable to presume that the mutants have a rigid structure which is fixed in a conformation allowing the C1q molecule to bind Fc. C1q binding to the rigid molecule may be energetically favourable compared to the flexible wild type molecule, as the loss in entropy upon complex formation is greater for a flexible molecule than for a rigid one. This may be the reason why the rigid molecules mediate complement lysis more efficiently than wild type $IgG_3$.

LH-2 , a Novel Lim-Homeodomain Gene Expressed in Developing Lymphocytes and the Central Nervous System

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We have isolated a novel Lim-Homeodomain gene, denotely LH-2, that has a pattern of expression distinct from that of related genes. The homeodomain of LH-2 protein is most related to that of the LIM/homeodomain proteins, particularly that of drosophila apterous gene. Outside of these regions, LH-2 has no obvious homology to previously charaterized proteins. In cell lines, expression of LH-2 was found primarily in B and T lymphoid cells. Expression in B lineage lines was greatest in lines that represented early stages of differentiation. In embryonic and adult tissues, the highest level of LH-2 expression was found in discrete regions of the developing central nervous system, primarily in diencephalic and telencephalic structures, and in a subset of primary and peripheral lymphoid structures. To investigate the functions of LH-2 in the development of lymphocytes and in the central nervous system, we have deregulated LH-2 expression in lymphocytes of transgenic mice and disrupted one allele of LH-2 locus in the ES (embryonic stem ) cells by homologous recombination.