ANTIBODY ENGINEERING: RESEARCH AND APPLICATION OF GENES ENCODING IMMUNOGLOBULINS

Organizers: Martin Rosenberg and Gordon Moore

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Theory and Practice of MAb Humanization

T 001 CONSTRUCTION AND *IN VITRO* CHARACTERIZATION OF A HUMANIZED MONOCLONAL ANTIBODY AGAINST *PLASMODIUM FALCIPARUM* SPOROZOITES, Daniel R. Sylvester, Mark R. Hurle, Carol Silverman, Timothy W. Theisen, Terence G. Porter, Subinay Ganguly, Michael Burke, Daniel O'Shannessy and Mitchell Gross, Division of Biopharmaceutical R&D, SmithKline Beecham Pharmaceuticals, King of Prussia, PA.

Although antimalarial agents have been used for decades, malaria continues to be a major world health problem. Currently over 20 million people travel to regions endemic for malaria and are increasingly at risk of malaria infection. This is due to the emergence of drug resistant strains of the lethal species of malaria parasite *Plasmodium falciparum*. One approach to provide short term prophylactic protection against malarial parasites would be to give passively an antibody capable of preventing sporozoite invasion into liver cells. The use of potent monoclonal antibodies directed against circumsporozoite surface protein, could achieve such passive protection. Unfortunately, easily derived mouse mABs have only limited use since they would elicit a human anti-mouse antibody (HAMA) response in man. To minimize the potential of a HAMA response it is possible to humanize the antibody using recombinant DNA techniques to insert the Complementarity Determining Regions (CDRs) of the murine mAB into a human variable region framework. We have constructed such humanized mABs and will present data describing the effects of mutations on the activities of the various humanized mABs.

T 002 MAKING RODENT ANTIBODIES AS IMMUNOSILENT AS HUMAN ANTIBODIES, William J Harris, Scotgen Biopharmaceuticals Inc, Research Centre, Kettock Lodge, Campus 2, Aberdeen Science and Technology Park, Balgownie Drive, Bridge of Don, ABERDEEN AB22 8GU, Scotland, U.K.

The in vivo human use of rodent monoclonal antibodies is severely limited by short half-life, poor effector function recognition and the human anti-mouse immune response (HAMA) which reduces the clinical effectiveness of repeated dosing. Effector function defects can be simply restored by replacing the rodent constant regions with human constant regions to provide a chimaeric antibody, while a number of techniques for CDR grafting to reshape human antibodies with the original specificity an affinity of the murine have been developed. These methods involve the retention of the murine hypervariable loops of the heavy and light variable chains accompanied by the replacement of specific amino acid residues within the chosen human frameworks to optimise the restoration of antigen binding affinity.

We have adopted a minimal modification approach of introducing as few non human residues as essential to restore antigen binding affinity. Data will be overviewed and discussed in terms of achieving a balance between the restoration of optimal affinity and the number of introduced murine amino acid residues. Data will be presented to suggest that in vivo biological activity can be equivalent even though there appears to be a several fold variation in affinity as measured by in vitro ELISA assays, suggesting that it may be preferable to sacrifice a degree of apparent affinity to reduce unnecessary modification of human frameworks

The majority of reshaped antibodies described to date have been based on the use of human frameworks defined from adult cell lines. Such frameworks all contain somatic mutations introduced during affinity maturation, some of which could give rise to immunogenic epitopes in other individuals or alter half-lives. To reduce this possibility we have reshaped antibodies using human germ line frameworks.

Evidence is now accumulating which suggests that a number of epitopes within framework regions may have biological activities such as cold agglutinin, mimicking superantigen etc. Such analysis suggests that care should be taken in selection of adult human frameworks and indeed in selection from human phage antibody repertoires, to avoid the inadvertent inclusion of epitopes which could adversely affect clinically significant parameters such as half-life and binding to normal human tissue.

T 003 CDR-GRAFTING AND VENEERING APPROACHES TO MAB HUMANIZATION. George E. Mark, III and Eduardo A. Padlan. Merck Research Laboratories, Rahway, New Jersey 07065 and Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, Maryland 20892

The past several years has seen the advent of numerous forms of recombinant antibody modifications and the inclusion of CDR-grafted and alternatively humanized rodent monoclonal antibodies to the traditional armamentarium of therapeutic drug substances. The common place MAb has been accompanied by the bi-functional MAb, the Fab, the F(ab')2, the Fv, the scFv, the dAb, and the pAb; eventually, single CDRs may find therapeutic utility. The clinical limitation common to all of these forms of binding pairs, should they be derived from a non-human source, is immunogenicity. This problem has been addressed by several laboratories using either sophisticated molecular modelling and/or simplistic primary sequence alignment searches for identifying acceptable human scaffolds into which the CDRs may be placed with the least compromise of their binding affinity. Subsequently, those constituent framework residues of the re-engineered molecules which may be responsible for sub-optimal ligand affinity are reverted to the original rodent residue.
The process of humanization will be exemplified through the re-engineering of the IB4 MAb whose use in acute inflammatory disease was found to be significantly restricted by its immunogenicity. This murine MAb has been humanized by grafting its complementarity determining regions into human variable region frameworks. The CDR-grafted IB4 retains most of its functional potency and all of its specificity. A novel approach to humanization, veneering, has also been applied to this MAb. By this procedure the skin of the murine antibody, specifically those residues accessible to surveillance by the immune system, is adjusted to appear human. The methodologies of antibody humanization and the functional and immunogenic properties of the resultant molecules will be described.

MAb Conjugates and Fusions

T 004 **FIBRIN-TARGETED RECOMBINANT HIRUDIN,** Christoph Bode[1], Marschall S. Runge[2], and Edgar Haber[3], [1]University of Heidelberg, [2]Emory University, and [3]Harvard School of Public Health, Boston, Massachusetts 02115

An important conclusion of the GUSTO trial was that early reperfusion correlated directly with improved survival, indicating that the development of more potent and specific plasminogen activators than those presently available may lead to enhanced clinical efficacy. With this goal in mind, we have previously shown that chemical conjugates or recombinant fusion proteins containing an antibody combining site specific for either fibrin (59D8) or for the platelet integrin $\alpha_{2b}\beta_3$ (7E3) and a plasminogen activator resulted in thrombus targeting and enhanced fibrinolysis. A recombinant protein comprising the Fab of 59D8 and the catalytic segment of scuPA was 5.6 times as potent as scuPA in thrombolysis in a baboon arterial thrombosis model, while effecting less α_2-antiplasmin consumption and a shorter prolongation of the bleeding time. We now examine whether the principle of thrombus targeting can be extended to an anticoagulant.

Recombinant hirudin was linked covalently to the Fab of fibrin-specific monoclonal antibody 59D8 (which selectively binds to an epitope on fibrin that only becomes exposed after thrombin cleaves fibrinopeptide B). Antibody-coupled hirudin bound to an immobilized peptide of the fibrin beta chain amino-terminal sequence and inhibited the peptidolytic activity of thrombin more efficiently than free hirudin. Thrombin inhibition dependent on binding to immobilized fibrin monomer was enhanced 1100 fold ($p<0.0001$). Hirudin–59D8 Fab was 10 times more effective than hirudin in inhibiting fibrin deposition on experimental clot surfaces in fibrinogen solution ($p<0.0001$) and human plasma ($p<0.0001$). The more effective inhibition of thrombin by the conjugate was supported by a significantly diminished concentration of fibrinopeptide A in the plasma supernatant of the clot. Inhibition of clotting by an uncoupled mixture of hirudin and 59D8 Fab was indistinguishable from that by hirudin alone, indicating that the conjugate's greater inhibitory activity was due to covalent linkage between antibody and hirudin. In vivo, in a baboon model, hirudin–59D8 Fab was 6.2 times more potent in inhibiting the growth of a thrombus than hirudin alone. Thus, fibrin-targeted hirudin shows considerable potential for effecting local anticoagulation while reducing the risks of systemic anticoagulation.

T 005 ANTI-HIV IMMUNOTOXINS, Seth H. Pincus, NIAID Rocky Mountain Laboratories, Hamilton, MT 59840

We have been using monoclonal antibodies to the HIV envelope (Env) protein(s) gp120 and gp41 to construct immunotoxins that might be used to treat HIV infection. Cells that are actively secreting HIV express viral antigens at the cell surface. By killing these cells before they can spread virus, the nidus of infection may be removed. The efficacy of these immunotoxins in eliminating HIV tissue culture infections has been demonstrated. Moreover, this efficacy demonstrates that within HIV-infected cells there is a recirculating pool of Env that reaches the cell surface and is then internalized. The internalization of this cell-surface Env is regulated in a number of ways which may be manipulated to enhance the efficacy of the immunotoxins. The ability of immunotoxins to become internalized and kill the target cell is not a direct function of the binding of the immunotoxin to the cell; antibodies to different epitopes on the same Env protein differ dramatically in their efficacy. We have created a panel of anti-HIV immunotoxins that are directed against conserved epitopes on Env and react with most HIV clinical isolates. These immunotoxins are ready for clinical testing. In the process of designing these immunotoxins, we have discovered several general principals that may be used to enhance the efficacy of immunotoxins to other, non-HIV, targets.

A number of potential problems loom for immunotoxin therapy, a major one being immunogenicity. Both the antibody and the toxin can induce an immune response. We are coupling antibodies to polyethylene glycol in an attempt to induce specific tolerance to the immunotoxin. If successful, such an approach would have important ramifications for the administration of any therapeutic protein.

T 006 EXPRESSION OF SINGLE-CHAIN Fv MOLECULES IN BACTERIAL AND MAMMALIAN SYSTEMS AND THEIR USE AS CYTOTOXIC TARGETING AGENTS, David M. Segal, Carolina R. Jost, Andrew J. T. George, Istvan Kurucz, and Julie A. Titus, Experimental Immunology Branch, National Cancer Institute, Bethesda, MD 20892.

A number of sFv molecules have been produced with the purpose of developing new reagents for targeting cellular cytotoxicity. sFv fusion proteins, containing a C-terminal peptide tag and having specificity for either DNP or the human transferrin receptor (TfR), were made in a bacterial secretion system. Most of the secreted products were insoluble, and after solubilization and refolding several mg. of active material were recovered, representing a small percentage of the total sFv produced. These sFv proteins, in conjunction with a bispecific antibody containing anti-CD3 linked to anti-peptide tag mAbs, were able to induce human cytotoxic T cells to specifically lyse target cells which expressed an appropriate cell surface antigen. In order to increase the efficiency of production of active sFv we have examined the expression of three sFv proteins, having specificity for DNP, TfR, and murine CD3, in COS-7 cells, using either a CMV or SV40 late promoter. Pulse-chase experiments showed that all three proteins were produced in relatively high amounts and were secreted into the medium. The anti-CD3 and anti-TfR sFv proteins were secreted almost quantitatively with about a 2 hr half time. Endo H studies of the anti-TfR sFv, which contains a single N-linked glycosylation site, suggested that the rate limiting step of secretion was the movement of the protein from the ER to the Golgi. The anti-DNP sFv was secreted more slowly than the others, but introduction of an N-linked glycosylation site at a position homologous to that of the anti-TfR antibody, greatly enhanced the rate and extent of secretion. All three of the secreted sFv proteins bound their antigens specifically, and where examined, all secreted material could be absorbed by the antigen. Therefore, the mammalian protein folding machinery is able to operate normally on the genetically engineered sFv fragments, producing, as far as we can tell, completely active protein. This may be of use in the production of complicated fusion proteins containing sFv portions, for example bispecific sFv proteins.

T 007 CHIMERISATION OF CELL SURFACE MOLECULES WITH ANTIBODY DOMAINS, André Traunecker, Klaus Karjalainen, Basel Institute for Immunology, 487 Grenzacherstrasse, Postfach CH-4005 Basel, Switzerland

The technology to produce soluble forms of cell surface molecules has been crucial for their structural and functional characterization, as well as for their exploitation as therapeutical reagents. The complex interactions between cells of the immune system usually involve large numbers of different surface molecules. The role of a single receptor-ligand pair can be understood by "solubilizing" these components from their natural cellular environments. These soluble molecules can then be used to dissect cellular interactions.

To illustrate the various principles of "solubilization" procedures of cell surface receptors, different versions of soluble recombinant CD4 (sCD4) molecules have been produced. Besides the natural function on the lymphocyte surface, CD4 is also the receptor for the human immune deficiency virus (HIV). Therefore, different generations of sCD4 were generated to maximize their potency as anti-HIV reagents.

Early versions of sCD4 consisted only of whole or partial extracellular domains of CD4. These molecules behaved as monomers in solution and importantly, they retained their high affinity for gp120, as well as their antigenic properties. In addition, they were shown to neutralize HIV efficiently *in vitro* . However, these simple forms turned out to be inefficient *in vivo*, presumably due to their extremely short biological half life.

Therefore, CD4 was chimerized to different effector moieties, which could then, in addition, mediate the destruction of infected cells. To this end, we and others have produced different forms of CD4-Ig molecules, where the CD4 moiety was joined to different Ig constant regions. These molecules were orders of magnitude more potent *in vitro* compared to earlier versions, presumably due to their multivalency, the most dramatic case being CD4-IgM. In addition, the constitutive expression of high levels of soluble mouse CD4-IgM in transgenic mice does not interfere with their immune function.

The newest version of sCD4 exploits cellular cytotoxicity as an effector arm. Those molecules, Janusins, are based on the principle of bispecific antibodies which were previously designed to retarget CTLs of any specificity onto desired target cells. To induce efficient killing in this system one antibody arm is specific for the constant part of the T cell receptor (TCR), e.g. CD3 ϵ chain and the other arm against the desired target epitope. Anti-HIV Janusin molecules are single polypeptide chains, which contain the CD4 and anti-CD3 ϵ antibody combining site (Fv CD3) as separate functional domains in the same molecule. These molecules can efficiently retarget CTL's onto HIV infected cells and induce efficent killing *in vitro* .

Catalytic MAbs and CDR Mimicry

T 008 SITE DIRECTED MUTAGENESIS OF A CATALYTIC ANTIBODY, Jon D. Stewart[1], Victoria A. Roberts[2], Michael W. Crowder[1], Elizabeth D. Getzoff[2], Stephen J. Benkovic[1], [1]The Pennsylvania State University, University Park, PA 16802, [2]The Scripps Research Institute, La Jolla, CA 92037.

Site-directed mutagenesis has been used to study catalytic antibody 43C9, which accelerates the hydrolysis of an aromatic amide by a factor of 10^6 over the rate of the uncatalyzed reaction *via* a multi-step mechanism involving an antibody-bound intermediate. A computer model of the antibody Fv fragment with bound antigen was constructed based on known structures in the Antibody Structural Database (ASD) and this model was used to guide site-directed mutagenesis experiments. These studies identified two residues that play key roles in the catalytic mechanism of 43C9: His L91 and Arg L96, whose side-chains act as a nucleophile and an oxyanion hole, respectively. In addition, the 43C9 computer model was used to design a Zn(II) binding site within the antigen combining site. The metal binding site, based on that of carbonic anhydrase, consisted of three histidine residues: an existing histidine at position H35 as well as two site-directed mutations (N-H33-H and Y-H95-H). The mutant 43C9 antibody bound Zn(II) with micromolar affinity and the binding of Zn(II) quenched the intrinsic antibody fluorescence, allowing this antibody to function as a biosensor for Zn(II). The binding site was highly selective for Zn(II) and the binding affinities for Mg(II), Ca(II), Mn(II), Fe(III), Co(II), Ni(II), Cu(II) and Cd(II) were at least two orders of magnitude lower. By measuring the absorbance spectrum of *p*-nitrophenol bound to the mutant antibody in the presence of increasing concentrations of Zn(II), we demonstrated that the antibody can simultaneously accommodate both Zn(II) and *p*-nitrophenol and that they are in sufficiently close proximity to interact electronically.

T 009 CHEMICAL AND BIOLOGICAL APPROACHES TO CATALYTIC ANTIBODIES, Kim D. Janda, The Scripps Research Institute, Departments of Molecular Biology and Chemistry, 10666 N. Torrey Pines Road, La Jolla, California 92037.

The generation of catalytic antibodies has been extensively documented. Fundamental in their procurement is the hapten's design, and exploitation of the immune system. To date, successful strategies in hapten design have centered around either a transition-state analogue approach or a methodology which we have termed "bait and switch catalysis". Efforts directed at probing the immune system for these catalysts have largely been accomplished via Kohler and Milstein's hybridoma technology. Numerous proposals have been put forth to obtain new and improved antibody catalysts; one of the most appealing is the screening of large combinatorial antibody libraries. Based on these natural and semi-synthetic libraries, it should be possible to access more sophisticated modes of antibody catalysis. The premise being that a greater diversity of binding/catalytic modes can be sampled in a rapid and efficient manner. The question thus becomes how can we abstract these potentially new forms of antibody catalysts? Conventional haptenic design is one possibility as it could provide improved catalysts; this based on the potentially greater statistical sampling array. Alternatively, one might be able to bias the antibody screening process towards methods which select directly for catalysis. In essence, redirect the assaying process away from simple binding, and instead, place emphasis on the direct identification of functional group catalytic machinery. We will describe a chemical mechanism based screening approach for the direct sampling of nucleophiles in an antibody binding site. Furthermore, we will show how this technique can be used to obtain antibody catalysts. A considerable amount of effort has been focused on the development of efficient catalysts for the synthesis or modification of complex molecules. With the emergence of catalytic antibodies, the potential of creating specifically tailored catalysts for synthetic transformations has become evident. Recently, we have focused our efforts on antibody catalysis in organic solvents in an effort to make catalytic antibodies more attractive catalysts from a synthetic standpoint. We have built a pneumatic device which allows for the automation of antibody catalysis in low water content media. The implementation of this robotic in a multigram synthetic scheme will be discussed.

Generation Display and Screening of Immunoglobulin Diversity I

T 010 ISOLATION AND CHARACTERIZATION OF IMMUNOTHERAPEUTIC Mabs FROM PHAGE DISPLAY LIBRARIES, Robert S. Ames, Mark A. Tornetta, Christopher S. Jones, Subinay Ganguly, Laurie L. Granger, and Ping Tsui, Departments of Molecular Genetics, Gene Expression Sciences and Protein Biochemistry SmithKline Beecham Pharmaceuticals, 709 Swedeland Rd. P.O. Box 1539, King of Prussia, PA 19406-0939.

Development of human Mabs has been hindered by the difficulty in isolating and maintaining stable human hybridomas. The advent of techniques for repertoire cloning and phage display of antibody fragments may facilitate isolation of therapeutically useful Mabs. We developed phage Fab display libraries to isolate antibodies reactive with the complement component C5a, Interleukin-5, and respiratory syncytial virus F-protein. Libraries were generated from spleen RNA isolated from mice that had been immunized with rC5a or rIL-5. They were enriched for specific Fabs via iterative biopanning versus antigen coated microtiter wells. Fabs reactive with C5a or IL-5 were identified by colony lift assay with iodinated ligand. The Fabs were further characterized by ELISA and receptor binding inhibition assays. Human peripheral blood leukocytes or spleen was the source of Fabs specific for F-protein. Limited availability of purified F protein precluded its use for Fab identification in colony lift assays. Following the last cycle of biopanning individual colonies were isolated, cultured in microtiter plates, and Fab expression was induced. An ELISA was used to identify colonies producing Fab. They were then screened for F-protein binding and virus neutralization. We have investigated various methods of purification of *E. coli* expressed Fabs, and have had some success using tag techniques, especially addition of poly-histidine residues coupled with chelate chromatography. Mammalian expression vectors were constructed and used to express full length immunoglobulin versions of Fabs isolated from the phage libraries. The ability to rapidly isolate Fabs from phage libraries and convert them to Mabs may mitigate the difficulties in developing human antibodies.

T 011 SYNTHETIC HUMAN ANTIBODIES, Carlos F. Barbas III, Jeff Smith, Dana Hu, Ben Cravatt, Jonathan Rosenblum, Wei-Ping Yang, Amelia Briones, Douglas Cababa, Jürgen Wagner, and Richard A. Lerner, The Scripps Research Institute, La Jolla, CA 92037.

Since your demonstration that libraries of human antibodies containing synthetic random CDR regions can be utilized to select antibodies which bind novel antigens using phage display, we have utilized this strategy to produce antibodies which bind and neutralize HIV and bind to human receptors including integrins with disassociation constants of 10-^{11}M. A strategy for the direct design and selection of anti-receptor antibodies has been developed. Furthermore, the synthetic strategy has been employed to evolve antibody affinity, to select metalloantibodies, to select for catalytic activity, and to create novel trivalent Fab' (mw 50kD) with homologous and heterologous binding activities.

T 012 **ANTIBODY ENGINEERING USING PARSIMONIOUS MUTAGENESIS**, James W. Larrick and Robert F. Balint. Palo Alto Institute of Molecular Medicine, 2462 Wyandotte St. Mountain View, CA 94043.

Antibody affinity is concentrated in only one-third of antigen-antibody contacts. We have developed a method called Parsimonious mutagenesis(PM) to engineer high affinity antibodies by mimicking the conservative process of natural selection[1]. In this process, high-affinity contacts are preserved while new ones are sought by conservative mutagenic scanning of the remaining contacts. Mutagenized V-regions are built up by PCR using primers which are synthesized with nucleotide mixtures in which the parent nucleotide at each CDR position is "spiked" or "doped" to predetermined levels with one or more of the other nucleotides. The level of spiking or doping determines the most important property of the library, that is, the average number of positions scanned at a time, or the "optimum scanning sequence". Sequences with this number of altered residues are the most abundant sequences in the library. For example, to improve an antibody with micromolar affinity one might expect 5 or fewer changes in the 16 or so potential contact residues of the light chain CDRs. In a PM library designed to scan 16 L CDR residues 5-at-a-time, sequences with 5 altered AAs comprise 21% of the library, and sequences with 1-5 alterations comprise 60% of the library. Thus, using doped nucleotide mixtures allows one to construct libraries in which the frequencies of the types of sequences most likely to contain improved variants are maximized. Regardless of the relative frequencies, though, the rarest substitutions in the library, with frequencies of 0.04%, would still be present in 40,000 sequences in a phage display library of 10^9 members. Thus, even the rarest substitutions in sequences with optimum proportions of non-parental residues are amply represented, even though the total sequence complexity of this library is $>10^{17}$, far beyond the reach of available cloning and/or screening methods. Error-prone PCR was used by two groups to generate antibody populations from which higher affinity variants were isolated. The reported mutation frequencies were used to compute the theoretical frequencies of the higher affinity variants in the libraries from which they were isolated. We also computed the theoretical frequencies of the same variants in libraries generated by PM. In both cases, the selected variants would have had significantly higher frequencies in PM-generated libraries. In one additional case, an oligonucleotide-directed codon-based mutagenesis method was used to remove an unwanted cross-reactivity from an antibody. The theoretical frequencies of the two best variants from that library were also compared to their frequencies in a PM library, and again, their frequencies in the PM library were significantly higher.

[1]Balint, RF, Larrick J.W. 1993. Antibody engineering using Parsimonious Mutagenesis. *Gene* (in press).

T 013 HIGH AFFINITY HUMAN ANTIBODIES ISOLATED USING PHAGE DISPLAY
Dr David J Chiswell - General Manager, Cambridge Antibody Technology Limited (UK)

The immortilisation of antibody gene rather than antibody producing cells and the development of large and diverse libraries displaying functional antibody fragments on phage has provided the tools to directly select antigen specific binding antibodies, revolutionising the techniques for making human antibodies.

Our latest progress on the isolation of human antibodies and their affinity maturation will be reported.

Generation Display and Screening of Immunoglobulin Diversity II

T 014 MAPPING AND ALTERATION OF ANTIBODY BINDING DETERMINANTS USING PHAGE DISPLAY, Jeffrey R. Jackson, Ganesh Sathe, Raymond Sweet, and Martin Rosenberg, Department of Molecular Genetics, SmithKline Beecham Pharmaceuticals, 709 Swedeland Road, PO Box 1539, King of Prussia, PA 19406

A high-affinity murine fab against human interleukin 1ß (IL-1ß) was subjected to mutagenesis of heavy chain CDR-3 (CDR-3H) and selection via phage display in an attempt to map binding determinants and increase the affinity. The codons of 6 amino acid residues in CDR-3H were individually randomized and the resultant libraries were selected separately by binding directly to immobilized IL-1ß. We identified single amino acid changes at each of these positions that reduced the affinity substantially (>>10 fold). The nature of these substitutions suggests that both the backbone conformation of the CDR-3 loop and certain side chains are essential for binding. Some residues, which may be involved in directing the conformation of the backbone, are very sensitive to substitutions, yet disruption of the canonical salt bridge at the base of the loop had little effect on affinity. Modest improvements in affinity were obtained via single amino acid changes at 2 positions near the top of the loop. A codon based mutagenesis strategy was then used to create multiple substitutions within the CDR-3H loop. This library was screened by several methods including competition with antigen or parental fab. Nevertheless, after several rounds of selection multiple CDR-3H sequences persisted in the selected phage, including the parental sequence, some of the single mutants identified above, and some double mutants with affinities equivalent to or slightly higher than the best single mutants. We conclude that CDR-3H plays an essential role in the function of this fab and the amino acid sequence of this loop in the parent molecule is nearly optimal. Further improvements in affinity, if possible, may be achieved by substitutions in other CDRs.

T 015 METAPHORIC ANTIBODIES AND PHAGEMID DISPLAY LIBRARIES, Bob Shopes, Stratacyte Corp., 11099 North Torrey Pines Road, La Jolla, CA 92037 USA

Human antibodies are preferred for therapeutic use but are difficult to obtain for most applications. A vast storehouse of potentially useful mouse monoclonal antibodies exists but induce an immune response in man. There is a need for human antibodies of the same efficacy. As an alternative to humanization, mouse antibodies may be transformed to fully human antibodies by a metaphoric process using phage display antibody libraries.

Generation Display and Screening of Immunoglobulin Diversity III

T 016 RECOMBINANT NEUTRALIZING HUMAN ANTIBODIES TO HIV-1, Dennis R. Burton[1], Carlos F. Barbas III[1], James M. Binley[1], Henrik Ditzel[1], Michael Hendry[2], H. Clifford Lane[3], Robert Walker[3], John P. Moore[4], Peter M. Nara[5], Erling Norrby[6], Markus Thali[7] and Joseph Sodroski[8], [1] The Scripps Research Institute, La Jolla, [2] California Institute of Public Health, Berkeley, [3] NIAID, Bethesda, [4] Aaron Diamond AIDS Research Center, New York, [5] National Cancer Institute, Frederick, [6] Karolinska Institute, Stockholm, [7] Dana Farber Cancer Institute, Boston.

Large panels of human antibodies to HIV-1 have been generated from libraries displayed on the surface of phage. The library donors were primarily long term asymptomatic HIV-1 seropositive individuals. Fab fragments reacting with the CD4 binding site, a site overlapping but distinct from the CD4 binding site, the V3 loop and several distinct epitopes on gp41 have been characterized. A number of Fab fragments from each category have been identified which show potent neutralizing activity against laboratory strains of HIV-1 in a number of assays. The most effective neutralizing Fab to the CD4 binding site, Fab b12, has been converted to a whole antibody. This IgG1 is found to neutralize MN and LAI strains of HIV-1 about 100 to 1000-fold more effectively, under comparable conditions, than a number of other CD4 site antibodies described in the literature and compared in the NIH Antibody Serology Project. Furthermore, the antibody is found to neutralize more than 50% of field isolates in a plaque assay. Engineering of Fab b12 by a mutagenesis/selection strategy has been carried out to improve the neutralization potency by more than an order of magnitude. Recent advances in developing new specificities, improving existing antibodies and understanding the molecular basis for potent neutralization will be presented.

T 017 EXPRESSION OF HUMAN IMMUNOGLOBULIN LOCI-DERIVED YACs IN MICE: TOWARDS MICE PRODUCING LARGE REPERTOIRE OF HUMAN ANTIBODIES, Aya Jakobovits, Larry L. Green, Margaret C. Hardy, Catherine E. Maynard-Currie, Hirohisa Tsuda, Donna M. Louie, Douglas Smith, Michael J. Mendez, Hadi Abderrahim, Masato Noguchi, and Sue Klapholz, Cell Genesys, Inc., 322 Lakeside Drive, Foster City, CA 94404.

Previous studies of the function of human immunoglobulin genes in mice, in which minigene constructs have been used, have suggested the value of employing large segments of the human immunoglobulin genes in their germline configuration for achieving high level of expression and diverse antibody repertoire. The ability to clone and introduce into mice extensive germline-configuration immunoglobulin gene arrays required the development of new technologies. We have developed a method for the introduction of large DNA inserts, cloned in Yeast Artificial Chromosomes (YACs), into the mouse germline via fusion of embryonic stem cells with yeast spheroplasts. Using this technology, large fragments of the human heavy and light chain loci were introduced into the mouse germline, including the germline of gene-targeted mice deficient in mouse antibody production. The application of this strategy to the generation of mice expressing significant levels and diverse repertoire of human antibodies, in the absence of mouse antibodies, will be presented.

T 018 HUMAN SEQUENCE ANTIBODIES FROM TRANSGENIC MICE. Nils Lonberg, Lisa D. Taylor, Condie E. Carmack, and Dennis Huszar. GenPharm International, 297 North Bernardo Avenue, Mountain View, CA 94043.

We have generated transgenic mice that contain human-sequence Ig miniloci and, because they are also homozygous for targeted disruptions of their endogenous heavy chain and κ-light chain genes, rely on the transgene sequences for B cell receptor expression. Although the human transgenes contain only a fraction of the intact human heavy chain locus, these defined sequences are able to at least partially restore the humoral immune system in the mouse. B cells expressing human receptors develop in the bone marrow, populate peripheral lymphoid tissue, and respond specifically to antigen. Furthermore, the heavy chain transgenes contain both human μ and γ1 coding exons as well as the respective μ and γ1 switch regions. The sequences included within the transgene are sufficient to direct class switch recombination. Transgene sequences are also sufficient to direct somatic mutation of the class-switched heavy chain genes. These transgenic mice are being used to generate human sequence monoclonal antibodies directed against human antigens.

Analysis of MAb Structure/Function via Genetic Manipulation

T 019 BIOLOGICAL PROPERTIES OF CDP571, AN ANTI-TNFα ENGINEERED HUMAN ANTIBODY.

Mark Bodmer. Celltech, Slough, UK.

There are many diseases for which a potential pathogenic role for TNFa has been identified. These include: sepsis and trauma induced shock; chronic inflammation such as in the rheumatoid joint; immune stimulation in organ allograft rejection; and metabolic disturbances resulting in cachexia in e.g. cancer patients. Clinical assay and animal model data suggest that clinical intervention to neutralise TNFa may be of therapeutic benefit. We have generated a recombinant human antibody (CDP571) engineered to bind to TNFa, and have investigated both the contribution of isotype to therapeutic efficacy in model systems, and the pharmacokinetics and immunogenicity in man.

Isotype selection of therapeutic antibodies may have important clinical consequences. In a rabbit model of rhTNF-induced pyrexia, a murine/human chimeric g4 anti-human TNF-a mAb (cCB0011) showed a dose-dependent inhibition of pyrexia, whereas a g1 isotype variant of the same mAb gave a marked pyrexia which was seen at all doses indicative of an immune complex mediated response. To investigate whether isotype difference could influence mAb efficacy in pathological disease states, hamster/murine chimeric g1 and g2a anti-murine TNF- mAbs (TN3g1, TN3g2a) were studied in experimental shock in mice and rats. In LPS-induced shock in mice, treatment with TN3g1 mAb at 30 and 3mg/kg resulted in 90% survival by 72 hours ($p=<0.004$), and prolonged survival to 45 hours ($p=<0.05$) respectively, compared to 100% mortality by 27 hours in controls. In contrast, a g2a isotype variant of the same mAb (30mg/kg) resulted in only 10% survival by 72 hours ($p=<0.05$). In a neutropenic sepsis model in rats there was greater survival in animals receiving the g1 isotype of TN3 compared to g2a isotype variant (70% vs. 27%; $p=<0.005$) with 100% mortality in the controls.

The pharmacokinetics of CDP571 have been investigated in an ascending-dose single administration study in healthy human volunteers at 0.1, 0.3, 1.0, 2.0, 5.0 and 10.0 mg/kg. The pharmacokinetic results show a long plasma residence time with a $bt_{1/2}$ of greater than one week and dose-proportionality across the six groups. The anti-CDP571 response in man was purely anti-idiotypic, peaked at 14 days and declined for the remainder of the study period. The CDP571 remained in the plasma during and beyond the period of the peak anti-idiotypic response, and retained its ability to bind TNF. Most of the response generated is IgM, and analysis of the patterns of IgM and IgG elicited suggest that CDP571 in man is much weaker at promoting the class switch to IgG than in monkeys.

T 020 T CELL-TARGETED IMMUNOFUSION PROTEINS FROM E. COLI, Marc Better[1], Patricia Nolan[1], Patrick Gavit[1], Dianne Fishwild[2], Hsiu-Mei Wu[2], Susan Bernhard[2], Sandra Soares[1], and Wilfredo Morales[1], Xoma Corporation, [1]1545 17th Street, Santa Monica CA 90404, and [2]2910 Seventh Street, Berkeley, CA 94710

Previous clinical experience has demonstrated that an immunoconjugate of the H65 antibody, which recognizes the CD5 antigen on the surface of human T cells, and the ricin A chain shows promise for the treatment of T cell-mediated diseases (1-2). Recent experience with antibody domains such as Fab, $F(ab')_2$, and single chain antibodies (SCA) has revealed that they can be produced from a microbial source such as *E. coli* at high yield, and that they also can be linked to ribosome-inactivating proteins (RIP) to generate highly cytotoxic immunoconjugates (3). To develop the most effective reagent for elimination of pathogenic human T cells, we constructed both chemically-crosslinked and genetically-fused conjugates between antigen-binding domains and RIP. Specific and cytotoxic reagents are produced by both approaches, yet direct expression of fully-assembled and properly-folded immunofusions as secreted proteins in *E. coli* offers a clear production advantage. The gene encoding the RIP gelonin was cloned and fused to the humanized H65 (he3H65) kappa, Fd or single chain antibody gene in various orientations. This family of fusion proteins, with gelonin at either the amino- or carboxy-terminus of the proteins, was produced by bacterial fermentation, purified and evaluated. The properties of various T cell-targeted immunofusions will be described.

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T 021 IMMUNOGENICITY OF VIRAL PEPTIDES GRAFTED IN SELF-IMMUNOGLOBULIN MOLECULES, Constantin A. Bona, Teodor Brumeanu, and Habib Zaghouani, The Mount Sinai School of Medicine, One Gustave Levy Place, New York, NY 10029

The advent of genetic engineering has allowed for the expression and production of recombinant proteins carrying short immunogenic epitopes of foreign antigens. These antigenized molecules represent valuable tools to investigate the molecular basis of antigen fragmentation, generation and presentation of peptide to lymphocytes, the induction of epitope specific immunity and the development of a new generation of vaccines. Recently, we expressed viral epitopes on Ig molecules by replacing the D segment of a V_H gene with a) influenza virus nucleoprotein epitope recognized by CD8 CTL (Ig-NP), b) influenza virus hemagglutinin epitope recognized by CD4 T-cells (Ig-HA), and c) a B cell epitope from V3 loop of HIV-1 envelop protein (Ig-V_3). The peptides generated from cells transfected with chimeric Ig-NP gene are recognized by CTL in a genetic restriction manner and exhibit an in vivo priming effect (1,2). Ig-HA vigorously activates HA specific CD4 T-cells. The magnitude of CD4 T-cells by Ig-HA is comparable to that induced by viral HA but is 300—1000 higher than that of synthetic peptide (3,4). Molecular analysis of peptides extracted from class II of APC pulsed with Ig-HA or influenza virus showed identical structure indicating that the viral epitope expressed in the CDR3 loop of self-immunoglobulin is generated into acid vacuoles like the viral HA (5). Finally, Ig-V_3 induced in baboon neutralizing antibodies which bind to gp120 protein and elicited in vitro production of antibodies when incubated with PBL lymphocytes from sero-negative patients without clinical disease. Our results suggest possible practical application for the development of prophylactic and immunotherapeutic reagents based on the utilization of antigenized immunoglobulins.

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T 022 SITE-SPECIFIC MUTAGENESIS OF THE CHROMOSOMAL IMMUNOGLOBULIN GENES OF MOUSE HYBRIDOMA CELLS: ANALYSIS OF VECTOR DESIGN AND GENE EXPRESSION, Diosdada Bautista, Adriana Oancea, Wenyong Sun, Jing Xiong, and Marc J. Shulman, University of Toronto, Toronto, Canada M5S 1A8

Homologous recombination between transferred and chromosomal Ig genes in mouse hybridoma cells offers a general method of altering the chromosomal Ig genes in predetermined ways. Recombination is infrequent in hybridoma cells, and we have endeavored to improve the methods for obtaining the rare transformants with appropriately modified κ or heavy chain loci.

In the case of the IgH locus, we have used a replacement-type vector bearing an enhancerless *gpt* gene; the absence of the enhancer greatly decreases the frequency of gpt^+ random transformants, whereas properly targeted transformants are gpt^+. The use of this simple enhancer trap allows recovery of transformants with the predicted recombinant structure at a frequency as high as 25% of the gpt^+ population. The recombinants express μ to the same extent, at a level comparable to that of the parental cell. The reproducibility of this modification indicates that it is reasonable to investigate the basis for high level expression by examining the effects of specific mutations.

The κ light chain locus behaves very differently. As for the IgH locus, we have used a vector bearing an enhancerless selectable marker (in this case the *neo* gene) and a segment encoding the human Cκ region, such that targeted recombinants produce mouse V/human C chimeric κ chains. When this vector is used in conjunction with the herpes thymidine kinase counterselection, homologous recombinants constitute 15% of the selected G418-resistant, FIAU-resistant cells. In analyzing the expression of the recombinant gene, we found that (a) the steady state level of chimeric κ mRNA is very variable among transformants with the same recombinant gene structure, (b) the variability in the amount of chimeric κ mRNA is much greater than the variability of the endogenous κ gene, (c) the variability in chimeric κ mRNA is much greater than the variability in the amount of *neo* mRNA, even though the *neo* gene is *in cis* with the chimeric κ gene and both depend on activating elements in the κ locus, (d) there is no systematic difference in the level of production by recombinants which retain or have lost the J-Cκ intron enhancer, and (e) the amount of chimeric κ mRNA in the highest producing transformants is much less than the amount of the corresponding mouse κ mRNA. These results suggest that expression of the chimeric κ gene is subject to strong epigenetic effects, which will seriously impede analysis of the effects of specific mutations.

Expression of MAbs and MAb Derivatives in Prokaryotic, Eukaryotic and Transgenic Systems

T 023 APPROACHES TOWARDS CANCER THERAPY BEYOND *NAKED* ANTIBODIES, Paul Carter[1], Brent Blackburn[2], Arthur Lee[2], Claire Kotts[3], Cindy Wirth[3], M. Refaat Shalaby[3], Len Presta[4] and Maria L. Rodrigues[1], Departments of [1]Cell Genetics, [2]Bioorganic Chemistry, [3]Medicinal and Analytical Chemistry and [4]Protein Engineering, Genentech Inc, 460 Point San Bruno Boulevard, South San Francisco, CA 94080.

Our humanized anti-$p185^{HER2}$ antibody, huMAb4D5–8 is currently in phase II clinical trials for metastatic breast cancer. We are currently using this humanized antibody as a building block in the construction of potentially more potent immunotherapeutics. Recent progress will be presented for a humanized bispecific antibody (anti-$p185^{HER2}$ / anti-CD3) designed to retarget cytotoxic T cells to tumor cells overexpressing $p185^{HER2}$. Another strategy being pursued is the use of antibody-enzyme fusion proteins for site-specific activation of prodrugs to drugs. For example, a fusion protein constructed between the β-lactamase RTEM–1 and a disulfide-linked Fv fragment was found to efficiently activate a cephalosporin prodrug of doxorubicin.

T 024 ANTIBODIES FROM PLANTS: ADVANTAGES AND DISADVANTAGES OF AGRICULTURAL PRODUCTION OF FOREIGN PROTEINS. Andrew Hiatt, The Scripps Research Institute, La Jolla, CA 92037.

Expression of cDNA constructs encoding full length mouse immunoglobulin chains with their native leader sequences, or fusion constructs substituting the native leader with a pre-pro sequence derived from *Saccharomyces cereviseae* yielded blocked N-termini on the γ chain or the correct amino terminal sequence on the mature κ chain. Lectin binding assays revealed that assembled immunoglobulin complexes contained a glycosylated heavy chain. The attached glycan was resistant to digestion by endoglycosidase H; its lectin binding pattern was distinguishable from the mammalian glycan due to an absence of terminal sialic acid. The results indicated processing of the immunoglobulin carbohydrate in the tobacco Golgi to yield a complex oligosaccharide. When purified, the tobacco-produced antibody was found to possess the antigen binding and specific catalytic properties of the original murine monoclonal antibody. Aglycosylated antibodies, Fab's, and single chain constructs have been efficiently expressed. The results in general show that the endomembrane system of plant cells possess cognate mechanisms for the recognition of diverse leader sequences; these signals can be used to initiate the assembly, processing, and secretion by plant cells of many complex foreign proteins. The advantages of producing complex proteins using agricultural methods include very low costs for production of large quantities; clonal propagation of desired plant lines; genetic stability of desired lines; assembly of subunits by cross-pollination; a wide variety of target organs and cell types for heterologous expression; an absence of human pathogens. The disadvantages primarily involve uncertainties about the potential biological effects of asialic glycans and an absence of precedents for validation of field or greenhouse production systems.

T 025 **IMPROVING THE FOLDING EFFICIENCY OF ANTIBODIES BY PROTEIN ENGINEERING.** **Achim Knappik[1], Konrad Bauer[2] and Andreas Plückthun[1]**, [1]Biochem. Institut, Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland. [2]GSF Forschungszentrum für Umwelt und Gesundheit, Haematologikum, Inst. für Immunologie, Marchioninistr. 25, D-81377 München, FRG.

Expression of antibody fragments in *E. coli* combines the advantages of powerful genetic manipulations and relatively simple large scale fermentation processes and therefore greatly enhances antibody engineering approaches [1]. Furthermore, the ability to functionally express correctly folded antibody fragments in *E. coli* is the prerequisite for any kind of functional screening of libraries. While a wide variety of antibody fragments have now been functionally expressed by secretion to the periplasm, the yields of this process have generally been only modest. There is evidence that frequently it is the periplasmic *in vivo* folding process itself, which is limiting the overall yield of folded protein [2]. One way to overcome this limitation is to use high-cell density cultivations [3,4], but we now wished to directly rectify the molecular causes in the primary sequence. For example, several fragments of the humanized antibody 4D5 are particularly well expressed [4]. We used this antibody and the murine antibody McPC603 in comparative studies, in which the amount of soluble, insoluble and functional protein was separately quantified, and sets of identical vectors were used.

We conclude that the differences in expression yields are entirely due to differences in the primary sequence. The expression yields of McPC603 are limited because (i) the antibody fragments aggregate in the periplasm and (ii) the cells start to lyse during induction of expression, while the same cells expressing 4D5 are stable. Using a site-directed mutagenesis approach, we identified several positions which are at least partially responsible for aggregation and lysis. Surprisingly, these two phenomena are *not* correlated, as *different* amino acids could be identified as causing them. One particular mutation greatly reduces the amount of insoluble protein and also leads to a three-fold increase in soluble antibody fragments, but does not affect the lysis phenomenon. A second mutation partially reduces the lysis phenomenon without, however, affecting the distribution soluble/insoluble in the periplasm. The two effects are additive: a combination of both mutations leads to a combination of both phenotypes. The corresponding antibody fragment gives 10-fold higher yields because aggregation is suppressed and a longer growth phase results from the reduction of the lysis phenomenon.

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T 026 HIGH LEVEL EXPRESSION OF MAbs IN CHO CELLS, Mitchell E. Reff, IDEC Pharmaceuticals Corporation, San Diego.

High level expression of immunoglobulin in CHO cells has resulted in cell lines that divide in less than 30 hours and secrete about 60-100 pg/cell day of immunoglobulin. Levels of >150mgs/liter have been achieved in spinner culture in 4 days with relatively low cell density (<2x10^6/cells/ml). The same cell lines produce >1 gram/liter in 11 day fermentation runs at low cell density. These high secreting CHO cells appear to be saturated for MAb secretion. Cells that contain more immunoglobulin heavy and light chain RNA on a per cell basis can be isolated, but they do not secrete any more MAb. High level expression of immunoglobulin in CHO cells is routinely performed by random integration of a plasmid that contains four separate transcriptional cassettes; neomycin phosphotransferase, dihydofolate reductase, immunoglobulin light chain, and immunoglobulin heavy chain. Non-immunoglobulin promoters and enhancers are used to express the immunoglobulin genes. The immunoglobulin genes in these vectors do not contain any splices, they are designed as cassette vectors with unique restriction sites which can accept immunoglobulin variable domains. After electroporation of the plasmid DNA into CHO cells, colony selection is used to isolate G418 resistant clones that are secreting between 5-10mg/liter/4 days. These high expressing G418 clones are usually single copy integrants of the plasmid. Novel strategies involving altering the neomycin gene will be discussed which decrease the number of G418 resistant clones that need to be screened to find these single copy clones that are very high producers. An individual high expressing G418 clone is then selected in 5nM methotrexate (MTX). Resistant colonies are screened for increased levels of MAb production. Individual colonies are selected in a second round of MTX (50nM) and again screened for high level production of MAb. The 50nM MTX CHO clones are usually secreting >100 mgs/liter/4 days of immunoglobulin. These cells have very low copy number (<10 copies/cell), and are extremely stable producers of MAbs.

T 027 IMPROVEMENTS IN HYBRIDOMA CULTURE AND BISPECIFIC ANTIBODY PRODUCTION, Duane Inlow, David Lowe, Bill Howarth, Heather MacDonald, David Harano, John Davis, Brian Maiorella, Mark Brannon, Dennis Fordham, Leo Lin, John Reeder, and David B. Ring, Chiron Corporation, Emeryville, CA 94608.

Large scale production of bispecific antibodies involves considerations common to the economical production of any monoclonal antibody, along with unique problems related to separating multiple immunoglobulin species that may share very similar physical properties. We have developed serum-free media and culture conditions that can increase antibody yield from hybridomas by over 20 fold. In our media, Pluronic polyol replaces serum in protecting cells from hydrodynamic stress, and lipid precursors (choline and ethanolamine) replace serum lipoproteins. Iron is supplied chelated with citrate, and the level of most amino acids is increased 10 fold to support increased cell growth and antibody secretion. Accumulation of inhibitory waste products is minimized by feeding glucose and glutamine in direct response to metabolic demand. With these conditions, we have achieved yields of 1 gm/l for a murine monoclonal antibody and 750 mg/l for a human monoclonal. Murine bispecific antibody 2B1, which targets Fcγ receptor III-bearing immune effector cells against c-erbB-2 positive tumor cells, provides a case study of special problems in bispecific antibody production. While some hybrid hybridomas produce up to 10 immunoglobulin species due to heterologous light / heavy chain association, 2B1 was chosen for development in part because such heterologous associations are disfavored, and only three species (bispecific and both parental antibodies) are formed. In this case, bispecific antibody could be separated from parental antibodies by anion exchange chromatography, but cation exchange revealed an unexpected inactive form of bispecific antibody that differs in glycosylation. A complete process for preparation of clinical grade bispecific antibody was developed based on sequential anion and cation exchange steps, and incorporating appropriate procedures for the inactivation or removal of murine retrovirus particles (incubation in Triton X-100 and incubation at low pH).

Clinical and Diagnostic Experience with Genetically Engineered MAbs

T 028 PERSPECTIVES OF CANCER IMAGING AND THERAPY WITH RADIOIMMUNOCONJUGATES. David M. Goldenberg, Center for Molecular Medicine and Immunology, Newark, NJ 07103

The targeting and imaging of cancers with radiolabeled antibodies (radioimmunodetection, or RAID) has developed over a period of 4 decades, with major advances made in the past 10 years. At the present time, murine and human whole IgG's and fragments labeled with In-111 or Tc-99m are in various stages of development or commercialization. These agents appear to be safe, with the major limitation being development of HAMA with murine MAbs, except when low doses of Fab' fragments are used. The major indications have been to complement other diagnostic tests and to disclose occult tumors. Third-generation Tc-99m/Fab' agents formulated by simple and direct labeling kits permit same-day imaging.

The potential for combining an imaging agent with a biological therapeutic for a more rational approach to detecting, staging, and treating a neoplasm is available with LL2, a MAb that recognizes the CD22 of the B-cell lineage. ImmuRAID-LL2 (Fab' labeled with Tc-99m) can differentiate tumor from scar tissue, permit restaging of extent of disease, provide a total-body survey of disease, and evaluate response to therapy. The whole IgG or $F(ab')_2$ labeled with I-131 (ImmuRAIT-LL2) has shown encouraging responses in patients with non-Hodgkin's lymphoma, at a divided, low-dose schedule (20+30 mCi doses). In this initial trial, a 40% response rate (CR+PR) was achieved, with the major side effect being myelosuppression. Methods to enhance the prospects of radioimmunotherapy will be discussed. (Supported in part by USPHS grant CA39841 from the NIH.)

T 029 **SPECIFIC PHOTOAFFINITY-LABELING LOCUS ON ANTIBODIES: USE FOR CHELATION, PROTEIN CONJUGATION AND GENE DELIVERY,** Heinz Kohler, Gabriela Pavlinkova, Krishnan Rajagopalan, H-T Wang, Sunil Chatterjee and Boyd Haley, Markey Cancer Center, University of Kentucky, Lexington.

Antibodies binding to cellular receptors often induce internalization of the complexed receptors. This property can be exploited to deliver biologically active molecules, such as toxins or genes, to target cells. The aim of this work is to develop mild chemical methods to conjugate proteins, nucleic acids and metal ions to antibodies. Using photoaffinity probes containing specific 8-azido purines or benzophenone we labeled purified polyclonal and monoclonal antibodies. Purified Fab fragments are also labeled without altering the antigen-specific binding. The photomodification is saturated at micromolar concentrations of probes indicating reaction with a distinct site on immunoglobulins. At saturation all detectable Ig is modified as determined by the complete protein shift in isoelectric focusing. The reaction is not restricted to a specific Ig isotype. Several purine and pyrimidine nucleoside and nucleotides provide competitive protection during labeling. Labeled antibodies show distinct isoelectric points which are shifted in comparison to unlabeled antibodies. The probes react with both heavy and light chains. However the labeling ratio of different antibodies is variable with different probes. These findings indicate that antibodies have distinct site(s) with high affinity for the heterocyclic base of purine nucleotides. These sites are readily accessible for photochemical modification. Collectively, these results demonstrate that photoactivated probes can be used to introduce chelating sites for metal isotopes, to conjugate polylysine for DNA complexing and gene delivery, and for generating protein conjugated antibodies. Strategies are discussed how to apply this technology to isotope labeling of antibodies for tumor imaging and for delivering DNA-complexed antibody to specific tumor cells.

T 030 GENETICALLY ENGINEERED MONOCLONAL ANTIBODIES IN THE CONTROL OF RESPIRATORY SYNCYTIAL VIRUS INFECTIONS, Geraldine Taylor, A.F.R.C. Institute for Animal Health, Compton, Berkshire, RG16 0NN, England.

Respiratory syncytial virus (RSV) is a major cause of respiratory disease in young children and occurs as annual winter epidemics. Infection with this virus results in high mortality in paediatric patients with underlying cardiopulmonary disease or with immunodeficiency and in immunosuppressed adults. Antibodies are known to mediate resistance to RSV infection and clinical trials of intravenous gamma globulin (IVIG), containing high levels of neutralising antibodies to RSV, in infants and young children have been effective both in the prevention and treatment of RSV infections (1,2). A significant reduction in the amount of immunoglobulin required for prophylaxis and therapy could be obtained with monoclonal antibodies (MAbs). Neutralising MAbs to the F protein of RSV that also inhibit fusion are the most potent antibodies identified so far, both in the prevention and treatment of RSV infection. A humanised MAb (RSHZ19) specific for the F protein has been engineered by transplantation of murine complementarity determining regions into a human IgG framework. RSHZ19 recognised all clinical isolates of RSV studied, was as effective as the original murine MAb at neutralising RSV *in vitro*, inhibiting giant cell formation and in the prevention and treatment of RSV infection in mice (3). RSHZ19 treatment on day 4 of infection had no discernable effect on the subsequent development of immunity to RSV. Furthermore, virus did not re-appear in the lungs of nude mice persistently infected with RSV, when RSHZ19 was administered on day 4 of infection, even when the antibody had declined to undetectable levels. Pharmokinetic studies have demonstrated that RSHZ19 has a long terminal half-life of 13 days in the monkey (4). These studies demonstrate that RSHZ19 shows great potential for use in the prophylaxis and therapy of RSV infections.

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Theory and Practice of MAb Humanization; MAb Conjugates and Fusions

T 100 MONOCLONAL ANTIBODY - SUPERANTIGEN FUSION PROTEINS: TUMOR SPECIFIC AGENTS FOR T - CELL BASED TUMOR THERAPY.

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The bacterial superantigen staphylococcal enterotoxin A (SEA) is an extremely potent activator of T lymphocytes when presented on MHC Class II molecules. In order to develop a tumor specific superantigen for tumor therapy, we have made a recombinant fusion protein of SEA and a F_{ab} reacting with human colon carcinoma cells. SEA as part of a fusion protein showed a reduction in MHC Class II binding by two orders of magnitude compared to native SEA and accordingly the affinity of the C215 F_{ab}-SEA fusion protein for the tumor antigen was about four orders of magnitude stronger than for MHC Class II molecules. The C215 F_{ab}-SEA fusion protein efficiently targeted T cells to lyse MHC Class II- negative human carcinoma cells, which demonstrates functional substitution of the MHC Class II dependent presentation of SEA with tumor specificity. Treatment of mice carrying B16 melanoma cells expressing a transfected C215 antigen resulted in 85- 99% inhibition of tumor growth and allowed long term survival of animals. The therapeutic effect was dependent on antigen specific targeting of the fusion protein. The results suggest that F_{ab}-SEA fusion proteins convey superantigenicity on tumor cells, which evokes T cells to suppress tumor growth.

T 101 GENERATION OF SOLUBLE IL-1 RECEPTOR BY CLEAVAGE AT A PROTEASE SITE ENGINEERED INTO THE HINGE REGION OF THE IMMUNOADHESIN IL-1R-IgG, Joanne Beck, S. Marsters, A. Ashkenazi and S. Chamow, Depts. of Recovery Process R&D and Molecular Biology, Genentech, Inc, 460 Pt. San Bruno Blvd., So. San Francisco, CA 94080. We sought a generic means by which any soluble receptor expressed as an immunoadhesin (a product of the molecular fusion of the ectodomain of a protein with immunoglobulin) could be purified, and the soluble receptor released by enzymatic cleavage as an intact Fd-like fragment, which could then be used for crystallographic and ligand-binding studies. To achieve this we selected genenase, a mutant form of subtilisin BPN', which exhibits greatly restricted substrate specificity. We introduced the preferred sequence for cleavage by genenase (AAHY:TF) into the upper hinge region of a human IL-1R immunoadhesin. Plasmid DNA containing the mutant sequence was used to transiently transfect embryonic kidney 293S cells. The secreted immunoadhesin was purified from cell supernatants by affinity chromatography on protein A- Sepharose. Genenase was immobilized on controlled-pore glass (CPG), using the heterobifunctional reagent MPBH. The initial rates of reaction with the synthetic peptide substrate sFAHY-*phenylnitroanilide* showed that the immobilized enzyme had activity that was indistinguishable from that of its soluble counterpart. Purified IL-1R-IgG was then incubated with CPG-genenase, and the products analyzed by SDS-PAGE and immunoblotting using anti-Fc and anti-IL-1R monoclonal antibodies. The specificity of the cleavage was confirmed by direct sequencing of the newly generated termini. The sIL-1R generated by digestion with genenase was separated from the Fc portion of the parent molecule by protein A chromatography. This approach offers a generic means by which any soluble receptor expressed as an immunoadhesin can be purified and enzymatically released as an intact Fd-like fragment for crystallographic or ligand-binding studies.

T 102 HUMANIZATION OF KC4G3, AN ANTI-HUMAN BREAST EPITHELIAL MUCIN ANTIBODY.

Joseph R. Couto[1], Eduardo A. Padlan[2], Edward W. Blank[1], Jerry A. Peterson[1] and Roberto L. Ceriani[1]. 1-Cancer Research Fund of Contra Costa, Walnut Creek, CA. 94596. 2-National Institutes of Health, Bethesda, MD 29892.

The murine antibody KC4G3 reacts with the majority of human carcinomas. We have previoulsy constructed the IgG_1,κ chimeric antibody version ChKC4G3 (Couto, J.R., et al., Hybridoma, 1993. 12(4): p. 485-489.]. We now report the successful construction of the IgG_1,κ humanized version HuKC4v2. HuKC4v2 was designed by using a positional consensus, of structurally important residues, previously developed after examining the 3-D structures of several antibodies (Padlan, E.A, Antibody Engineering. 1992. San Diego, CA.: IBC). We previously showed that some κ chains of KC4G3 and ChKC4G3 migrate abnormally on SDSPAGE due to glycosylation. We detected an N-linked glycosylation signal in V_κ of KC4G3 (Couto et al., ibid) that was purposely deleted in HuKC4v2. Thus, all humanized κ chains migrate normally on SDSPAGE. Both the affinity and the ability to compete for antigen binding were completely preserved in HuKC4v2. The HuKC4v2 frameworks conform to the V_κII and V_HIIId human consensus in all but six positions in V_κ and three positions in V_H. Three of the murine residues that were retained (in these positions) exist in greater than 24% of the sequenced human antibodies. Of the remaining residues, 2 probably have buried side chains and 2 others probably contact the opposite domain. Thus, all 4 may be unavailable for binding to B-cell receptors.

T 103 ISOLATION OF SINGLE-CHAIN VARIABLE DOMAINS WITH HIGH-AFFINITY ANTIGEN-BINDING CHARACTERISTICS USING THE TWO-HYBRID SYSTEM IN YEAST.

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The development of non-animal based methods of antibiody production has become increasingly favored in recent years. In addition, the development of methods of producing human-derived or humanized versions of murine-derived antibiodies has parallelled the attempts to develop the non-animal systems. Concurrently, other groups of investigators studying primarily signal transduction pathways and/or transcriptional regulation have developed several methods for assessing the protein/protein interactions inherent in these systems. Our current studies that will be described combine the technological advances and existing needs of both of the aforementioned groups to produce a genetic selection system in yeast that is based on a transcriptional readout assay of reporter gene function with the goal of isolating immunoglobulin variable domains with high affinity for desired antigens. The system is based on the two-hybrid system described originally by Fields and Song (Nature 340:245, 1989). The variable domains in the transactivation domain fusion library can be derived from human or murine sources, and although the initial experiments have been directed towards isolating single chain variable regions with antigen binding properties, this system can be utilized to isolate combinatorial antigen binding domains. The selected variable regions of interest can be subsequently cloned into human or murine immunoglobulin expression vectors and produced in large amounts as desired. The variable domains that have been targeted to date have been directed towards signal-responsive transcriptional regulatory factors of the Cyclic AMP Response Element Binding protein/Activating Transcription Factor (CREB/ATF) family. The goal of these initial studies has been to target fusion molecules containing the antigen-recognizing variable domain as a chimeric protein with a strong constitutive transcriptional activating domain to endogenous DNA-bound transcription factors that are unstimulated under basal conditions. In this manner, it is hypothesized that we will be able to investigate whether certain members of transcriptional regulatory protein families are endogenously bound to and potentially regulating specific promoters *in vivo*. Supported by NIH grants GM45872 and DK47120 and ACS grant BE71630 (to JPH).

T 104 CONSTRUCTION OF AN ANTI-CEA scFv FRAGMENT WITH Tc-BINDING PROPERTIES FOR USE IN TUMOR IMAGING.

Wolfgang E. Höhne[+], Jens Schneider-Mergener[*], Reinhard Malin[*], Susann Koch[+], Clemens Lill[+], Helga Wessner[+], Renate Steinbrecher[#], and Wolfhard Semmler[#], [+]Institut für Biochemie, [*]Institut für Medizinische Immunologie, der Humboldt-Universität Berlin, D-10098 Berlin, [#]Institut für Diagnostik-Forschung am Klinikum Virchow der FreienUniversität Berlin, D-14050 Berlin, Germany

It was shown that the murine anti-CEA monoclonal antibody B4FC3 binds specific to carcinoembryogenic antigen (CEA) but did not bind to nonspecific cross-reacting antigen (NCA). The antibody recognizes a conformational epitope of the protein part of the highly glycosylated CEA as demonstrated by binding studies with deglycosylated CEA. Unfolded or proteolytically degraded CEA was not recognized by the antibody. In addition, synthetic peptides derived from CEA sequence regions predicted to be antigenic or overlapping peptides covering one of the highly homologous repetitive regions did not bind to B4FC3 at all.

Using the amino acid sequence of the light and heavy chain variable regions deduced from the base sequence, a structural model was derived starting with Fab fragments from the Brookhaven Protein Data Bank. The features of the binding area formed by the six CDR loops are discussed.

The specific accumulation of the antibody B4FC3 as well as its Fab fragment in tumors could be demonstrated in nude mice bearing a human colon carcinoma xenograft (LS 174T). The Fab fragment showed a faster elimination from blood and therefore yielded higher tumor to blood ratios, if compared with the whole antibody.

In addition, the immunogenicity of the antibody should be low. This is why single-chain Fv fragments are usually thought to be especially suited for this purpose. For that reason several single-chain Fv fragments of the B4FC3 were constructed linking the heavy chain and the light chain variable regions with a $(GGGGS)_3$ peptide linker. The recombinant protein was expressed in E.coli. One construct contained a leader sequence for secretion into the periplasmatic space, another did not, resulting in formation of cytoplasmatic inclusion bodies. The yield was considerably higher if the scFv fragment was purified from the inclusion bodies applying an optimized refolding strategy.

To load the scFv fragment with the radioisotope technetium for tumor scintigraphy Tc-complexing peptides were fused to the C-terminus of the polypeptide chain by extending the coding gene fragment with a corresponding synthetic oligonucleotide. Those Tc-complexing peptides were designed using a novel cellulose-bound combinatorial hexapeptide library XB_1XB_2XX (X = 19 genetically encoded amino acids, Lys omitted) and subsequent iterative screening for single Tc-complexing peptides.

Antibodies raised against these peptides were used to detect the expression products. The CEA and Tc-binding behaviour of the corresponding single-chain Fv fragments was tested.

T 105 Humanized Antibodies to Respiratory Syncytial Virus: Production and Preclinical Evaluation, S. Johnson, G. Bansal, F. Brady, D. Couchenour, S. Dillon, M. Dormitzer, D. Feller, D. Goldman, J. O'Grady, D. Pfarr, C. Riggin, J. Tamura, MedImmune, Inc., Gaithersburg, MD, and G. Prince, Virion Systems, Inc., Rockville MD.

Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract infection in young children. In the United States alone, approximately 90,000 children are hospitalized and 4,500 children die from RSV infection each year. At special risk of serious RSV morbidity and mortality are children with underlying diseases such as congenital heart disease, broncho-pulmonary dysplasia and other pulmonary diseases, various congenital or acquired immunodeficiency syndromes and prematurity. There are over 100,000 such high risk children in the United States. Currently the most promising approach to prophylaxis of RSV disease in high risk infants is passive immunization. We have generated humanized versions of two murine monoclonal antibodies which neutralize RSV at low concentrations. Both computational and empiric methods were used. Both of these humanized MAb's have affinities close to that of the parent molecules. Both retain the neutralizing capacity of the parent MAb's. Data pertaining to the production and preclinical testing of these antibodies will be presented.

T 106 HUMANIZATION OF A MOUSE MONOCLONAL ANTIBODY DIRECTED AGAINST P-SELECTIN.

S. Tarran Jones, José Saldanha, Margaret E. Cronin, Dolores Crowley, Alison L. Levy, and Mary M. Bendig, MRC Collaborative Centre, Mill Hill, London, NW7 1AD, UK.

Carl Perez, Leslie Walker, and Michael Kriegler, Cytel Corporation, San Diego, California 92121, USA.

The cell adhesion molecule P-selectin appears to play a critical role in reperfusion injury by recruiting neutrophils to ischaemic tissue following reperfusion. Mouse monoclonal antibodies (Mabs) against P-selectin could have a useful role in the inhibition of this neutrophil migration which often results in permanent damage to the reperfused tissues. Mouse Mabs, however, are immunogenic in human patients thus limiting their use as therapeutic agents. In order to circumvent this problem, a mouse Mab raised against P-selectin was humanized in two ways. A chimeric antibody was constructed that contained the variable regions from the mouse antibody and constant regions from human antibodies. In a more complete humanization process, only the complementarity determining regions (CDRs) of the mouse Mab were grafted into human variable regions. The humanized variable regions were then joined to human constant regions to create the "reshaped human" antibody. This latter method of humanization is more complex and it can be difficult to recreate a good antigen binding site. In order to assist in the design of functional CDR-grafted variable regions, a structural model of the mouse variable regions was built. DNA sequences coding for the reshaped human antibodies were constructed and expressed in mammalian cells. The mouse, chimeric, and reshaped human (CDR-grafted) antibodies were analyzed and compared in antigen-binding assays. All three antibodies had comparable affinities for antigen. The fully humanized (CDR-grafted) anti-P-selectin antibody is being developed for clinical testing.

T 107 GENERATION AND FUNTIONAL CHARACTERIZATION OF A HUMANISED ANTI-CD28 MONOCLONAL ANTIBODY, Klaus Kühn, Alan P. Lewis, Herman Waldmann* and J. Scott Crowe, The Wellcome Research Laboratories, Beckenham, Kent, UK and *Dept. of Pathology, University of Cambridge, UK. Humanised monoclonal antibodies may represent a powerful therapeutic tool for various human diseases, including autoimmune diseases like vasculitis and rheumatoid arthritis. We have generated 2 humanised versions of a rat antibody (YTH 912.13) with specificity for CD28 (clustered as anti-CD28 at the 4th Leukocyte Workshop, Vienna, 1989). CD28 is a T-lymphocyte receptor molecule involved in the initiation of the second signal of T-cell activation and plays a crucial role in the induction of T-cell anergy in vitro. For humanisation the light and heavy chain of the rat antibody were engineered to have homology with the "best fit" human light and heavy chain frameworks. The light chain of the antibody was humanised by PCR grafting of the CDRs onto the light chain framework of a human subgroup II antibody. The heavy chain was humanised by in vitro mutagenesis of selected framework residues thereby generating a human subgroup I framework. In addition to a completely humanised version of the heavy chain a partially humanised version was constructed in which the framework III residues 66, 67, 69 and 71 were left as the original rat amino acids. The humanised heavy chain variable regions were fused with the human γ1 constant region and together with the humanised light chain were expressed in a transient expression system. Results of functional studies as well as modelling data will be presented.

T 108HUMANIZATION OF A MOUSE MONOCLONAL ANTIBODY FOR THE TREATMENT OF MULTIPLE SCLEROSIS.

Olivier J. Léger, Simon Keen, José Saldanha, S. Tarran Jones, and Mary M. Bendig, MRC Collaborative Centre, Mill Hill, London NW7 1AD, U.K.

In an animal model for multiple sclerosis (MS), scientists at Athena Neurosciences (South San Francisco, CA) have shown that antibodies directed against $\alpha 4$-$\beta 1$ integrin can block the adhesion of leukocytes to the endothelium thus preventing inflammation in the central nervous system and subsequent paralysis in the animals. Athena scientists have isolated a mouse monoclonal antibody (mouse 21.6 antibody) directed against $\alpha 4$-$\beta 1$ integrin that is being developed for therapeutic use in humans. This mouse antibody has been fully humanized at the MRC Collaborative Centre. The mouse antibody was humanized by grafting the complementarity determining regions (CDRs) from the mouse 21.6 variable regions into human variable regions that were homologous to the mouse variable regions. In addition, a few amino acid residues in the framework regions (FRs) of the selected human variable regions were changed. A molecular model of the structure of the mouse 21.6 variable regions was built to assist in the design of the humanized variable regions. Based on the model, as well as information on the structure of immunoglobulin variable regions, certain residues were identified as having key roles in antigen binding and thus were retained during the design of the humanized variable regions. A fully humanized 21.6 antibody was designed and constructed that bound to cells expressing $\alpha 4$-$\beta 1$ integrin as well as mouse or chimeric 21.6 antibodies. This antibody had the six CDRs from mouse 21.6 antibody, four amino acid changes in the FRs of the human light chain variable region, and five amino acid changes in the FRs of the human heavy chain variable region.

T 109 A SINGLE AMINO ACID SUBSTITUTION WITHIN H3 IMPROVES BINDING ACTIVITY OF THE BR96 SINGLE-CHAIN ANTIBODY FRAGMENT 5-8 FOLD.

Stephen J. McAndrew, Scott Glaser†, Simone M. Webb, Jürgen Bajorath, Dale Yelton and Wesley L. Cosand. Bristol-Myers Squibb Pharmaceutical Research Institute, 3005 First Avenue, Seattle, WA 98121 and †Ixsys Inc., San Diego, CA 92121

The murine monoclonal antibody (mAb) BR96 recognizes a Lewis-Y (Le^y)-related antigen and reacts strongly with most human breast, colon, lung and ovarian carcinomas. We have cloned the BR96 variable (V) region genes and constructed a 28 kD single-chain antibody fragment (sFv). BR96 sFv was expressed in E. coli, refolded in vitro and purified to near homogeneity. The refolded material retained tumor specificity for cells bearing Le^y-related antigen and showed slightly reduced binding than the Fab in an ELISA-based format.

Previous work using codon-based mutagenesis of the BR96 Fab expressed in M13 phage display libraries has identified a mutation in BR96 that improves its K_D to Le^y. The low expression levels of Fab produced from phage makes accurate determination of affinity constants difficult. By expressing the BR96 mutation as a plasmid-encoded sFv, we are able to conveniently produce 10-20 mg/L of refolded protein. These quantities have made it possible to study the binding kinetics of BR96 and mutant sFv's to a synthetic Le^y-HSA conjugate using surface plasmon resonance. Using this technique, we are able to measure the kinetic rate constants, k_{on} and k_{off}, and calculate the equilibrium constant. In comparison to the proteolytically-derived BR96 Fab, the sFv has a 4-fold higher K_D. The mutant sFv, incorporating a single amino acid substitution within H3, has a K_D which is improved 5-8 fold, primarily due to a decreased k_{off}. A homology-based molecular model of BR96 Fv shows that this antibody has a distinct groove-type binding site which contains a significant number of aromatic residues. The effects of this mutation on K_D will be considered with respect to the molecular model.

T 110 DESIRABLE PROPERTIES OF ANTIBODIES FOR IMAGING AND THERAPY, Geoff A. Pietersz, Wenjun Li, Kenia Krauer, Vivien Sutton, Terry Baker*, Ian F.C. McKenzie, Austin Research Institute, Austin Hospital, Studley Road, Heidelberg Vic 3084, Australia, *Celltech Ltd, 216 Bath Road, Slough SL1 4EN Berkshire, UK.

Over the last few years, when monoclonal antibodies/immunoconjugates have been used for imaging and therapy, various parameters have been examined to determine which are the optimal characteristics for deciding whether antibody should go to the clinic. Such decisions are not trivial, and whereas most of the preclinical testing is straightforward, clinical testing is not, and is extremely expensive. It is therefore important that clear parameters be delineated for taking reagents from the laboratory to the clinic. Here we have not examined antibody specificity or class, but have examined other parameters which emerged after extensively testing our breast cancer BC2 (anti-MUC1) antibody which performed satisfactorily in histological and other studies, but was found to be lacking in imaging and therapeutic studies and chimaeric anti-MUC1 antibodies. A comparison was made between murine anti-MUC1 antibody BC2 (which react with the peptide epitope APDTR) and the "humanised" antibody hCTMO1 from Celltech which reacts with the MUC1 epitope RPAP. Preliminary studies demonstrated that CT101 was "good" antibody, whereas BC2 was not. Various parameters and conclusions reached were:- a) Affinity. The affinity hCTMO1 was 4.03 x 10^{-9} and of BC2 was 2.92 x 10^{-9} - we did not consider these numbers to be substantially different, although hCTMO1 was clearly higher affinity than BC2. b) On/Off rate at 4°C. Both antibodies bound effectively to the MUC-1 transfectant Mor-CF5; the association rate for hCTMO1 was x3.8 that of BC2 and the dissociation rate for BC2 was x2 faster than that of hCTMO1. c) On/Off rates 37°. At 37°C the ratio of association and dissociation rates for BC2 and hCTMO1 was similar to 4°C. d) Internalization. hCTMO1 was also more efficient in internalizing bound antibody; 70% of bound hCTMO1 was internalized, while <10% of bound BC2 was internalized. From these studies it was clear that while hCTMO1 was of similar affinity to BC2, the faster uptake and internalization and lower off rate indicated it was likely to be a superior antibody; this was proven *in vivo*. e) Localisation. hCTMO1 bound much better *in vivo* than BC2 (37% vs 17%) 37% while BC2 was 17%. f) Therapeutic experiments. BC2 idarubicin conjugates were essentially ineffective in irradicating tumours in mice whereas hCTMO1- idarubicin had a dramatic effect on breast cancer tumour cells growing in mice. We conclude that the simple measurements of On/Off rates and internalisation at 37°C are the most important parameters to use to determine antibody effectiveness, prior to embarking on clinical studies.

T 111 DEVELOPMENT OF A METHOD TO PRODUCE CHICKEN ANTIBODIES, Nancy M. Michael, Mary Ann Accavitti and Craig B. Thompson, Department of Medicine and Molecular Genetics and Cell Biology, Howard Hughes Medical Institute, University of Chicago, Chicago, IL 60637 and the Division of Rheumatology, University of Alabama at Birmingham, Birmingham, AL 35294

Our laboratory has a long term interest in avian B cell development. Unlike mammals, chickens do not use combinatorial diversity to generate the immunoglobulin repertoire. Instead, chickens generate diversity in the immunoglobulin loci by sequential gene conversion during B cell development within the bursa of Fabricius. We are taking advantage of the distinctive characteristics of chicken B cell biology and recent technological advances in an attempt to develop a method to efficiently produce chicken monoclonal antibodies. Since the chicken will make antibodies to proteins that are highly conserved in mammalian species and often not antigenic in the mouse, we hope to make monoclonal antibodies that will be useful biological reagents with research, diagnostic and therapeutic applications. We will present various design strategies and report on our progress.

T 112 LABORATORY-SCALE PRODUCTION AND BIOLOGICAL CHARACTERIZATION OF BR96 sFv-PE40, A POTENT SINGLE-CHAIN IMMUNOTOXIN CAPABLE OF REGRESSING TUMOR XENOGRAFTS, Leland Paul, Chris Phillips, Beth Evans, Carole Spangler, Dennis Fait, Dana Chace and Clay Siegall, Bristol-Myers Squibb Pharmaceutical Research Institute, 3005 First Avenue, Seattle, WA 98121

We have produced an immunotoxin fusion protein, BR96 sFv-PE40, that preferentially targets tissues expressing a Lewis Y related antigen. Many breast, lung and other carcinomas display high levels of this antigen on the cell surface. The fusion protein is composed of the antigen combining region of monoclonal antibody BR96 linked to the translocation and catalytic domains of *Pseudomonas* exotoxin. Intravenous administration of BR96 sFv-PE40 can regress and cure established human breast carcinoma xenografts in athymic mice and rats.

One of the fundamental problems faced in testing and analyzing this and other single-chain immunotoxins is simple and consistent production of sufficient high quality compound. Expression in *E. coli* results in deposition of the fusion protein into inclusion bodies which necessitates solubilizing and refolding to recover active material. We find that initial purification in the denatured state simplifies analytical characterization of refolding. Ion exchange chromatography in urea-containing buffers allows isolation of reduced monomeric protein that can be refolded by dilution. After refolding, ion exchange chromatography effectively resolves aggregates and the desired monomeric product. The process stream and final product are analyzed by several techniques including size exclusion HPLC and cytotoxic activity *in vitro*.

A laboratory-scale process yields in excess of 3 mg active immunotoxin per liter of culture. Mutant constructs, including higher affinity analogs, have also been produced with the developed protocol. Improved capacity to produce biologically active compound has allowed exploration of safety and efficacy in larger animals and may lead to human clinical trials with BR96 sFv-PE40.

T 113 ENZYME LABELLING OF A MURINE ScFv ANTIBODY VIA THE USE OF GENE FUSION, Richards G.R. and Walsh T. P., Centre for Molecular Biotechnology, Queensland University of Technology School of Life Science, Brisbane GPO Box 2434, AUSTRALIA

The gene for the enzyme firefly luciferase (Luc) has been attached to the 5' end of a gene encoding a murine single chain Fv (ScFv). Results will be described using the construct in *Escherichia coli* to produce a bifunctional protein. The concomitant expression of antigen binding and enzyme activity will be analysed. Genetic engineering of the construct has been undertaken to evaluate the effects of variable linkages on the two activities of this protein. Preliminary experiments using different enzymes may also be included.

Work aimed at using the recombinant protein in rapid tests for inflammatory response factors will be presented. Other potential applications of such a protein include its use in immunoassays, biosensors, and in the study of antigen-antibody interactions. Further work is being undertaken to determine its usefulness in the above areas and also the construction of further gene fusions with antibody fragments.

T 114 HUMANISED ANTIBODIES SPECIFIC FOR HUMAN CANCERS P.Wallace[1], K. Armour[1], F. Carr[1], M. Fitzek[1], S. King[1], K. Kitamura[2], W Rettig[2], S. Welt[2], L. Old[2] and W. Harris[1], [1]Scotgen Biopharmaceuticals Inc., Research Centre, Kettock Lodge, Campus 2, Aberdeen Science and Technology Park, Bridge of Don, Aberdeen, AB22 8GU, Scotland, [2]Ludwig Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA.

The in vivo use of murine antibodies for the diagnosis and therapy of human cancers is limited by their short half life in human serum, by the adverse HAMA response and by poor Fc dependant stimulation of human effector functions. These deficiencies can be overcome by the conversion, using recombinant DNA methods, of murine antibodies to 'humanised' antibodies. The production will be described of cancer-specific humanised antibodies by the transfer of murine complementarity determining regions and a minimal number of murine framework residues to the human frameworks NEWM or KOL (for the heavy chain) and REI (for the light chain). In each case, the humanised antibody retains the antigen specificity of the original murine antibody and binds the antigen with equivalent (or near equivalent) efficacy. These novel antibodies are potentially useful pharmaceutical agents for the in vivo diagnosis and treatment of human cancers.

Antibody Engineering: Research and Application of Genes Encoding Immunoglobulins

Catalytic MAbs and CDR Mimicry; Generation Display and Screening of Immunoglobulin Diversity I

T 200 DESIGN OF CATALYTIC ANTIBODIES FOR CARBOHYDRATE SYNTHESIS, Wenling Dong[1], Tina M. Jespersen[2], Mikœl Bols[2], Troels Skrydstrup[3], Michael R. Sierks[1], [1]Chemical and Biochemical Engineering Department, University of Maryland Baltimore County, Baltimore, MD 21228; [2]Department of Organic Chemistry, Technical University of Denmark, Lyngby, Denmark; [3]Institute of Organic Chemistry, C.N.R.S., Gif S/Yvette Cedex, France.

Carbohydrates play an important role in many biological processes. The ability to hydrolyze or synthesize specific carbohydrate linkages is therefore very desirable. Phage display techniques expressing the human immunological repertoire provide a powerful means to generate antibodies having specific carbohydrase activity. Diverse libraries containing human light chain and heavy chain variable domains are constructed utilizing polymerase chain reactions (PCR) of a human B-cell lymphocyte cDNA library. A random combinatorial library of single chain variable domains, obtained by combining a light chain, linker DNA, and a heavy chain, is then cloned and displayed on the surface of the bacteriophage geneIII minor coat protein. Chemically synthesized transition-state analogs will be used as antigens to select for strong binding antibody fragments. The chemically synthesized pseudo-monosaccharide, (3R,4R,5R)-3,4-dihydroxy-5-hydroxymethyl-piperidine hydrochloride (Figure 1) and pseudo-disaccharide, methyl 6,7-dideoxy-7-((3R,4R,5R)-3,4-dihydroxy-5-hydroxymethyl-piperidinyl)-α-D-gluco-heptopyranoside hydrochloride, (Figure 2) will be tested as antigens. The inhibitory capabiilities of these compounds will be compared with other known inhibitors including acarbose, deoxynojirimycin, and D-gluconolactone. Compounds with low K_d values will be used as haptens to screen the phage library for strong binders using affinity chromatography.

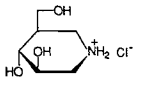


Figure 1

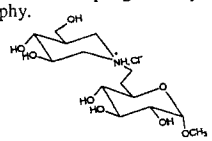


Figure 2

T 201 GENERATION OF LARGE PHAGE ANTIBODY REPERTOIRES USING *IN VIVO* RECOMBINATION, Igor Fisch*, Andrew Griffiths* and Greg Winter*§. *MRC Centre for Protein Engineering and §MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.

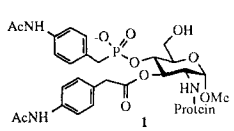
The use of rodent monoclonal antibodies has been a powerful diagnostic and research tool over many years, but as therapeutic agents, they have not fulfilled their initial potential. The main handicap of rodent monoclonal antibodies is the antiglobulin response and the hypersensitivity they produce once injected into man. Therefore, in order to bypass these limitations, antibody fragments, comprising paired heavy (VH) and light (VL) chain variable domains, can be displayed on the surface of filamentous bacteriophage, and rare phage (encoding antigen binding activities) selected by binding to antigen. The size of phage antibody repertoires (10^8) is limited by the efficiency of transformation of *E. coli*. Fragments with a range of binding specificities have been isolated without immunisation, with binding affinities in the range $10^5\ M^{-1}$-$10^7\ M^{-1}$. However larger "primary" repertoires of phage antibodies should allow higher affinity fragments to be isolated. In principle, larger repertoires could be made by combinatorial infection, for example by transforming *E. coli* with a repertoire of heavy chains (encoded on plasmids) then infecting with a repertoire of light chains (encoded on phage). Since infection is extremely efficient, and most *E. coli* cells in an exponential culture can be infected, the combinatorial diversity of Fab fragments displayed on phage could be as large as the number of *E. coli* in culture (10^{11} per litre). However the heavy and light chain genes would not be packaged together within the same phage particle, and so could not be simultaneously co-selected. We have created a system, based upon the *lox*-Cre site-specific recombination system of bacteriophage P1, to lock together the heavy and light chain genes from two different replicons within an infected bacterium and thereby create phage antibody repertoires at least three orders of magnitude larger than any previously available. Using this system we have created a 6.5 x 10^{10} repertoire of phage antibodies. The heavy chains, kappa light chains and lambda light chains in this repertoire were synthesised *in vitro* by combining human germline V-gene segments, with J-segments and constructing randomised CDR3 sequences. We have now isolated from this single repertoire antibodies against haptens, human antigens and 'foreign' antigens. The effect of increased repertoire size on affinity will be discussed.

T 202 REGIO- AND STEREOSELECTIVE DEPROTECTION OF ACYLATED CARBOHYDRATES VIA CATALYTIC ANTIBODIES, Ikuo Fujii, Yoshiharu Iwabuchi, Hideaki Miyashita, Ryuji Tanimura, Keiko Kinoshita and Masakazu Kikuchi, Protein Engneering Research Institute, 6-2-3 Furuedai, Suita, Osaka, 565 Japan

Considering the inherent high binding specificity of antibodies, catalytic antibodies in which the substrate specificity can be programmed by the hapten could provide powerful tools for regio- and stereoselective organic synthesis. In particular, regioselective protection and/or deprotection of carbohydrates in oligosaccharide synthesis are interesting targets for testing the potential of catalytic antibodies. Although many efforts have been mounted to develop regio- and stereoselective syntheses of oligosaccharides, the problem of differential protection of potentially competing functional groups is still formidable. Herein, we report the generation of catalytic antibodies that are capable of discriminating between chemically identical functional groups in the same molecule, catalyzing regioselective deprotection of acylated carbohydrates.

While a spontaneous hydrolysis of diester **2** gave an equilibrium mixture (4 : 1) of 4-OH and 3-OH due to acyl migration, an antibody raised against phosphonate **1** catalyzed the hydrolysis of **2** at C-4 with high regio- and stereoselectivity, affording 4-OH exclusively, despite accompanying acyl migration. In addition to these selectivities, the antibody demonstrated applicability for a wide range of substrates (ex. β-isomer at C-1). Thus, These antibody-catalysts should greatly simplify the conventional, complex synthetic procedures for oligosaccharides.

To investigate the substrate specificity, the 3D-structure model was constructed based on primary amino acid sequence of the Fv fragment. We also discuss a mode of molecular recognition of the catalytic antibody.



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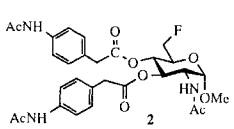


2

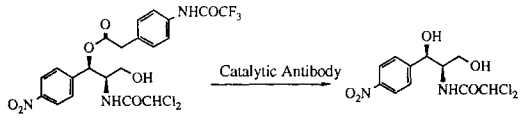
T 203 IN-CELL PCR APPLICATIONS; NON-RANDOM COMBINATORIAL LIBRARIES OF HUMAN ANTIBODIES AND DETECTION OF HIV-1 TRANSCRIPTS AT THE SINGLE CELL LEVEL, Guy Gorochov+, M. J. Embleton*, P.T. Jones+ and Greg Winter*+, Laboratory of Molecular Biology+ and Centre for Protein Engineering , MRC centre, Hills road, Cambridge CB2 2QH, U.K.

We previously described a process for the amplification of mRNAs in single cells. In a model system, it was shown that the Ig heavy and light chain V-genes (VH and VL) of murine hybridoma cells could be specifically amplified and self-assembled *in situ*, thus retaining the original pairing of those genes. Fluorescently labelled PCR products could be visualised intra-cellularly, and specifically, in hybridoma cells Similarly, untransformed human cells are now being used as templates for in-cell PCR in order to clone natural antibody repertoires and to study gene expression at a single cell level. In order to generate antibody repertoires, several type of cells from various sources have been tested. The best results are obtained with *in vitro* polyclonally stimulated peripheral B cells or with freshly isolated tonsilar plasmocytes. Cells are fixed, permeabilised and their RNA content reverse transcribed. We designed a set of primers that allows up to 3 successive nested PCRs and that covers all V gene segments described. In-cell assembly of VH to VL can be achieved by PCR overlap or by incorporating Lox-P sites in the VH and VL PCR products in a first round PCR followed by an incubation with *cre* recombinase in order to link the two products. The cells are washed after the first PCR and the linked products are reamplified in the cells with nested primers. Finally, after another washing step, nested primers are used to append restriction sites, to allow in-cell amplification of a clonable product encoding an antibody fragment in the form of a single chain Fv suitable for bacterial expression. We succeeded in obtaining librairies that are now being screened against common antigens. As said above, in-cell PCR can also be used for detection of messenger RNAs. Rare messenger species like those encoding for the regulatory proteins of the HIV-1 virus can be amplified after only 35 cycles of PCR, the products being labelled during the process by incorporation of fluorescent dUTP molecules. The cells can then be analysed by conventional or confocal microscopy and by flow cytofluorometry. The method was used to monitor the proportion of infected cells present in the blood and the lymphoid organs of HIV patients, like others (Patterson *et al., Science* 260, p.976, 1993), we found that this proportion was much higher than previously evaluated with less sensitive techniques.

T 204 PRODRUG ACTIVATION VIA CATALYTIC ANTIBODIES, Hideaki Miyashita, Tomoko Hara, Ryuji Tanimura, Masakazu Kikuchi, and Ikuo Fujii*, Protein Engineering Research Institute 6-2-3 Furuedai, Suita, Osaka 565 Japan

Prodrug activation via antibodies was examined using the antibiotic chloramphenicol as a model drug. Antibodies elicited against a phosphonate transition-state analog were found to catalyze the hydrolysis of a non-bioactive chloramphenicol monoester as a prodrug at a significantly higher rate above the uncatalyzed background reaction to regenerate chloramphenicol as a parent molecule. The antibody-catalyzed prodrug activation was tested by the paper-disc diffusion method using *Bacillus subtilis* as an indicator strain. The antibody (6D9) catalyzes the reaction with multiple turnover to generate enough chloramphenicol to inhibit bacterial growth, as indicated by a clear inhibitory zone after incubation with monoester. In contrast, no inhibition was detected by incubation of either the monoester or the antibody alone. This result reveals that only the antibody hydrolytically activates the monoester, which can be expected to be a suitable prodrug, as it is resistant to the action of bacterial hydrolytic enzymes.

To investigate the structure-activity relationship, we examined cloning of cDNA encoding Fab fragment of antibody 6D9. cDNAs prepared from the hybridoma were amplified by PCR. PCR fragments were inserted to lambda phage vectors, and then each heavy and light chain genes were coupled. The lambda phage DNA was converted to plasmids by an auto excision system. Fab fragment of 6D9 was produced in *E.coli* having these plasmids. Binding activity of Fab fragment secreted into the medium was confirmed by ELISA. Based on the 3D molecular models of CDRs, we discuss the correlation of catalytic activity with the antigen combining site structure.



"Prodrug" Monoester (inactive)

"Parent drug" Chloramphenicol (active)

T 205 STRATEGIES FOR THE PRODUCTION OF CATALYTIC ANTIBODIES USING COMBINATORIAL LIBRARIES. Lynda J. Partridge, Juqun Shen, S-X. Deng, Anthony Heaton, Adriana Borriello, Dennis R. Burton and G.Michael Blackburn, Krebs Institute, Departments of Chemistry and Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2UH.

Despite some successes, there have been problems associated with the production of catalytic antibodies. Whilst this undoubtedly relates in part to the difficulties of accurately predicting enzyme mechanisms and synthesizing suitable hapten analogues, the limited diversity of antibodies produced by conventional techniques may also be a factor. Combinatorial library methods of generating antibodies are potentially much more versatile. In the pComb3 vector system (Barbas et al. (1991) Proc. Natl. Acad. Sci USA 88 7978) combinatorial libraries in the form of antibody Fab fragments are displayed on the surface of M13 phage particles. Phage expressing antibodies with the desired specificity can be selected by affinity binding and plasmids derived from these phage particles used to produce soluble Fabs in *E.coli*.

Our laboratory is using both hybridoma and combinatorial library systems to generate candidate abzymes. Transition state analogues (TSAs) for the amide hydrolysis of the antibiotic chloramphenicol have been used for immunisation. A combinatorial phage display library (10^6 members) has been generated in the pComb3 vector from an immunisation with a sulphonamide TSA. Soluble Fabs from ten clones that bind specifically to the TSA have been isolated and are being tested for catalytic activity. Similar approaches have been adopted to generate catalytic antibodies for the *cis-trans* isomerisation of prolyl peptides.

T 206 A GENETIC APPROACH TO THE GENERATION OF ANTIBODIES WITH ENHANCED CATALYTIC ACTIVITIES, Phillip A. Patten, Helle D. Ullrich, Nathaniel S. Gray, and Peter G. Schultz, Department of Chemistry, U.C. Berkeley, Berkeley, CA 94720

Catalytic antibody technology has provided a general route to first generation catalysts. However, the rate enhancements obtained with catalytic antibodies are typically two to several orders of magnitude below those of the corresponding highly evolved enzymes found in Nature. This presumably reflects limitations in hapten design, limits on accessible antibody divesity found in vivo and the fact that immunological selection is based on binding rather than on catalysis. We have developed a catalytic antibody based microbial selection with the goal of evolving antibodies with improved activity through mutagenesis and selection in *E. coli*. for improved catalysis. We have cloned the genes for the model catalytic antibody 48G7, a biotin nitrophenyl esterase, and have developed an efficient *E. coli* expression system that secretes 1-5 mg/liter of active antibody into the periplasmic space. We are currently selecting libraries of mutants for improved activity in an E. coli biotin auxotroph. Additionally, we have cloned the germline genes utilized by 48G7 and we are using site directed mutagenesis to examine the functional roles played by somatic mutations fixed during affinity maturation of this antibody.

T 207 GENERATION AND COMPARISON OF SINGLE-CHAIN (sFv) ANTIBODY FRAGMENTS SPECIFIC FOR HUMAN CD7, Mary E. Pauza, and Christopher A. Pennell, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455

Recombinant immunotoxins (IT) can be constructed by linking genes that encode sFv antibody fragments to toxin genes. Our goal is to construct recombinant sFv-IT specific for CD7, an antigen expressed by the majority of human T cell leukemias. Since the binding characteristics of an IT can affect its efficacy, we first sought to generate a panel of CD7-specific sFv fragments to select ones with high affinities and different epitope specificities. A sFv library was generated using bacteriophage display as follows. Male C3H mice were immunized i.p. or i.v. with 2 x 10^7 CEM cells (a human T lymphoblastoid cell line) on days 0, 14, 28, and 31. Sera and splenocytes were harvested on day 34. Six of 10 mice produced significant amounts of CD7-specific serum antibodies as evidenced by ELISA results using a soluble human CD7-receptor globulin (Rg) fusion protein (mean $OD_{405}=1.092\pm 0.139$) versus soluble human VCAM-1-Rg (mean $OD_{405}=0.275\pm 0.047$) and human Ig (mean $OD_{405}=0.195\pm 0.005$). The splenocytes from these six mice were pooled and mRNA isolated. RT-PCR was used to generate V_H and V_κ libraries, which were then joined with a linker by splice overlap extension to yield a sFv library. The sFv fragments were expressed on phage as fusion proteins with the g3p coat protein. A comparison of epitope specificities and relative affinities of the CD7-specific sFv fragments obtained from selected phage will be presented.

T 208 CDR MIMICRY WITH ANTIBODIES AGAINST THE EXTRACELLULAR INTERFERON γ RECEPTOR

John A. Robinson†, Ashley Birch†, Angela Bridges†, Nicole Ruegg†, Geoffrey Williams†, Michel Aguet§, Gianni Garotta*, Walter Huber* and Daniel Schlatter*, † Institute of Organic Chemistry, and § Institute of Molecular Biology I, University of Zürich, 8057 Zürich, Switzerland; * Dept PRT, F. Hoffmann-La Roche, 4002 Basel, Switzerland.

The study of monoclonal antibodies (mAbs) that inhibit the binding of human *interferon* γ (IFNγ) to its cellular receptor may prove useful for the design of novel, small molecule IFNγ antagonists. In earlier work, several mAbs were identified that block the ligand binding site on the extracellular domain of this receptor. Competition binding assays and epitope mapping with expressed receptor gene fragments, indicate that 14 mAbs compete with each other, as well as with IFNγ, for the receptor, and most likely recognize overlapping epitopes in the putative N-terminal Ig-like domain. One other mAb does not compete with these 14, and appears to bind a region within the second putative Ig-like extracellular domain. Using radioimmunoassays as well as surface plasmon resonance, biosensor-based affinity measurements, the K_d's of the 15 mAbs (which span the range 1-80 nM), and the k_{on}- and k_{off}-rate constants, for the receptor have been measured. For selected mAbs, PCR-based cloning methods have allowed sequencing of the variable regions cDNAs. From the deduced variable region protein sequences, the CDRs have been produced as synthetic peptides. Competition binding assays with the immobilized recombinant extracellular domain of the IFNγR, and with the intact receptor on Raji cells, have been used to identify peptides that block the binding of either the parental antibody, or IFNγ, to the receptor. Attempts to optimize the affinity of such CDR-mimics for the target antigen, using combined chemical and biological approaches are underway.

T 209 DISPLAY OF CDR-LIKE PEPTIDES MOUNTED ONTO A MINIBODY SCAFFOLD, Sollazzo M., Toniatti C., Salvati A.L., Venturini S., Pessi, A., Tramontano, A., Cortese R., Ciliberto G., Martin F., I.R.B.M.- P. Angeletti, via Pontina km. 30,6 - 00040 Pomezia, Italy.

If peptides could be constrained conformationally onto a suitable small protein scaffolding, allowing the prediction or the determination of their conformation by NMR techniques, would give a considerable boost to the drug discovery process. In fact, this would open the door to pure chemical applications (Saragovi *et al.* 1992). We recently described the design and chemical synthesis of the minibody, a 61-residue β-protein with a novel fold (Pessi *et al.*, 1993). The aim of the design was to transfer onto a smaller molecule some desirable properties of immunoglobulins, namely (i) tolerance to sequence variability in selected regions of the protein, the hypervariable regions, and (ii) predictability of the main chain conformation of these loops, based on the "canonical structures"model. We constructed a library of "minibodies" ($20x10^6$ individual clones) with randomized loop sequences displayed on the surface of the g3p protein of *fd-tet* coliphage. To test the phage-minibody library as a potential source of constrained ligands, we used Human recombinant IL-6 as a ligate. Human IL-6 constitutes a suitable model system as the IL-6 receptor belongs to the Ig superfamily, thus its fold is related to that of the minibody. We have selected several clones that appear to interact with Human IL-6. A few interesting candidates have been characterised in detail.

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Saragovi, H. U., Greene, M. I., Chrusciel, R. A., Kahn, M.(1992) *BioTech.* **10**, 773

T 210 KINETIC, MOLECULAR CLONING, AND MODELING STUDIES OF A CATALYTIC ANTIBODY WITH CARBAMATASE ACTIVITY, Carston R. Wagner, Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455

Several examples of antibodies carrying out the hydrolysis of esters and amides have been observed, but not of urethanes or ureas.[1] Consequently, we have investigated the ability of the well characterized antibody MOPC-167 to hydrolyze carbamates of choline chloride. Ascites fluid containing MOPC-167 was obtained and the antibody purified by affinity chromatography. The potential substrate, p-nitroaniline carbamate to choline chloride was synthesized by condensation of para-nitroaniline isocyanate with choline chloride in the presence of triethylamine, and purified by ion-exchange chromatography. Michaelis-Menten parameters were obtained spectrophotometrically for the conversion of the carbamate to PNA , by monitoring the increase in absorbance at 404 nm. The values for k_{cat} and K_m for carbamate hydrolysis were found to be comparable to carbonate hydrolysis.[2] To facilitate mutagenic experiments, the Fv portion of the antibody was cloned, constructed into a single chain antibody, and a molecular model built with the software package AbM. (American Cancer Society, NIH (CA61908)

(1) Lerner, R. A.; Benkovic, S. J.; Schultz, P. G. *Science* **1991**, *252*, 659-667.
(2) Pollack, S. J.; Schultz, P. G. *Cold Spring Harbor Symp. Quant. Biol.* **1987**, *52*, 97-104.

Generation Display and Screening of Immunoglobulin Diversity II & III

T 300 PRODUCTION OF ANTI-CEA PHAGE ANTIBODIES: SINGLE CHAIN Fv FRAGMENTS DISPLAYED ON FILAMENTOUS PHAGE. Sarah M. Andrew, Juliet C. Beavis, Jessica B. Radin, Susan S. Heath, Penny M.K. Milling. Biological Sciences Division, Lancaster University, Lancaster, LA1 4YQ, England, UK.

Single chain Fv fragments consist of the variable domains of the immunoglobulin (Ig) heavy and light chains joined by a linking polypeptide. Their display on filamentous phage libraries allows selection of proteins which bind specifically to the antigen of choice at an early stage in production.

cDNA was obtained from the splenocytes of mice immunised with carcinoembryonic antigen (CEA). This was amplified using two sets of oligonucleotide primers, one hybridising with heavy chain variable Ig sequences, and one with light chain variable Ig sequences. The primers also encoded the central linking peptide, consisting of (gly-gly-gly-gly-ser)3, restriction enzyme sites, and a hexa-histidine tag for purification of the protein by metal affinity chromatography. A second round of amplification was performed on a mixture of heavy and light chain DNA fragments obtained in the first round. In this second round only the extreme 5' primer of the heavy chain DNA, and the 3' primer of the light chain DNA, were present. The resultant combined product formed a single chain of DNA encoding, from 5' to 3': an SfiI site, heavy chain variable domains, the central linking molecule, light chain variable domains, six histidines, an amber stop codon, and a NotI site. The cut product was ligated into the phagemid vector pHEN, which contains the PelB leader sequence, and the coding region of a phage coat protein, g3p. The construct was electroporated into the *E. Coli* suppressor strain XL1 Blue, and phage expression was induced. Precipitated phage were subjected to rounds of selection on immobilised CEA to identify phage expressing anti-CEA sFv on their gene3 protein. A negative selection round was carried out to exclude non-specific binding. The selected phage were used to infect a non-suppressor strain of *E.Coli* , TOPP2, for production of single chain Fv fragments with specific binding to CEA.

T 301 A MODEL OF THE COMBINING SITE OF A RECOMBINANT ANTIBODY TO ATRAZINE, Christopher W. Bell[1], Sunil Maulik[2], Victoria A. Roberts[3], Vernon K. Ward[4], and Alexander E. Karu[1], [1]U.C. Berkeley Hybridoma Facility, Albany CA 94706, [2]Oxford Molecular Ltd., Palo Alto CA 94303, [3]The Scripps Research Institute, La Jolla, CA 92037 and [4]Antibody Engineering Lab, U. C. Davis, CA 95616.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) and its analogs are among the most heavily used herbicides worldwide. The Fab region of a triazine-binding $IgG_{2b}k$ monoclonal antibody (AM7B2) was cloned and the H and L chain DNA sequences were determined by V. K. Ward, et al., (*Protein Engineering 6,* in press, 1993). The combining site was modeled by the combined-algorithm method using AbM™ v1.2, energy minimized using GROMOS, and visualized with ProExplore™ (Oxford Molecular Ltd). The overall quality of the model was assessed using PROCHECK™. The model was compared with a database of superimposed crystallographic structures, leading to the rebuilding of the L CDR1 (15 residues long). There is a Tyr-23 at L FR1-CDR1 boundary instead of the highly conserved Cys found in similar antibodies, which forms an intramolecular disulfide bond. In the light chain, Tyr-32 and His-34 of CDR1 and Ile 91 and Thr-95 of CDR3 form part of the triazine binding site. In the heavy chain, the side chains of Tyr-33 and His-35 of CDR1, Trp-50 and Tyr-52 of CDR2 and Leu-95 extend into the site. Asp-97 and Glu-99 of H CDR3 may also extend into the binding site. The model suggests that atrazine binding is predominantly hydrophobic, with some electrostatic interactions. Work to correlate this model with lipophilicity, electron shell properties, and relative binding of various triazines is in progress. (Sponsored by Dept. of Energy subcontract to AEK).

T 302 GENERATION OF COMBINATORIAL ANTIBODY LIBRARIES BY SIMULTANEOUS ANNEALING OF HEAVY AND LIGHT CHAINS TO A M13 TEMPLATE, Joseph Buechler, Diane McGrath and Gunars Valkirs, Biosite Diagnostics, San Diego, CA 92121

An improved method has been developed for cloning antibodies to a specific antigen where restriction enzymes are not used. In this method, RNA from mice hyperimmunized with antigen was isolated from the spleen. After cDNA synthesis, the antibody chains were amplified by polymerase chain reaction procedures using 24 heavy chain 5' PCR primers and 1 heavy chain 3' PCR primer, and 27 kappa light chain 5' PCR primers and 1 kappa light chain 3' PCR primer. The 5' PCR oligos contain a 20 nucleotide sequence complementary to an M13 uracil template, and the 3' PCR oligos contain antibody constant region sequences also complementary to the M13 template. After the preparation of double stranded PCR products, single stranded-DNA (ss-DNA) was prepared by assymetric PCR using a fraction of the double stranded PCR products as templates. The ss-DNA from each heavy chain reaction or each light chain reaction were pooled, concentrated and purified by HPLC. This ss-DNA was then used to mutate an M13 uracil template containing complementary sequences to the ss-DNA so that the heavy and light chains were simultaneously annealed to the template. After electroporation of DNA into bacteria, a library of M13 phage expressing antibody on the surface of the phage coat was made. This phage library was then screened for antibodies exhibiting a desired affinity and specificity.

T 303 ISOLATION AND CHARACTERIZATION OF NUCLEIC ACID-BINDING ANTIBODY FRAGMENTS FROM AUTOIMMUNE MICE-DERIVED BACTERIOPHAGE DISPLAY LIBRARIES, Michael J. Calcutt, Thomas P. Quinn, and Susan L. Deutscher, Department of Biochemistry, University of Missouri, Columbia, MO 65212.

The display of antibody fragments (Fab) on the surface of filamentous bacteriophage and the selection of phage that bind to a particular antigen has enabled the isolation of Fab with numerous specificities, including haptens, proteins and viral particles. We have extended this application by isolating nucleic acid-binding Fab from a combinatorial library of phage displaying Fab derived from the antibody repertoire of autoimmune (MRL/lpr) mice.

One of several Fabs isolated has been affinity purified to greater than 95% and characterized in more detail. The Fab demonstrated high affinity binding of various ssDNA molecules and competition analysis revealed a marked preference for poly-dT. Several model RNAs could also be bound. The heavy chain variable region was derived from a VH gene that is used recurrently in autoantibodies and was most similar to an anti-ssDNA autoimmune monoclonal antibody. This suggests that antigen-binding specificities present in an autoimmune repertoire may be directly accessed by this approach.

Results from light chain shuffling experiments showed that antigen specificity could be retained with several alternative light chains. These findings together with analyses of mutant Fabs containing amino acid replacements in the putative antigen binding site will be discussed.

T 304 ANTIBODY FRAGMENTS TO PRP GENERATED USING PHAGE DISPLAY TECHNOLOGY, Martin J. Cann and Jeff W. Almond, Department of Microbiology, University of Reading, Whiteknights, Reading, Berkshire, UK.

PrP^{sc} is the abnormal form of the normal host cell protein, PrP^{c}. PrP^{sc} is the major, if not the only, component of the infectious agent causing the Transmissible Spongiform Encephalopathies such as Scrapie, Bovine Spongiform Encephalopathy, and Creutzfeld-Jacob disease.

The PrP proteins of the different animal species susceptible to the infectious agents of the Transmissible Spongiform Encephalopathies, which include sheep, cows, humans, and mice, show a high degree of amino acid homology and this has restricted the use of hybridoma technology in generating monoclonal antibodies to PrP. Very few of the monoclonal antibodies raised by hybridoma technology have been able to distinguish between the PrP proteins of different species and none have been able to distinguish between PrP^{c} and PrP^{sc}.

For these reasons the phage-antibody technology developed by Greg Winter and colleagues is being exploited to generate antibody fragments against PrP. A naive human IgM library has been screened against *Escherichia coli* produced recombinant bovine PrP and a primed library is being constructed using purified bovine PrP^{sc} isolated from infected cow brains. Results concerning the properties of isolated phage will be presented and discussed.

T 305 **CELL AFFINITY ENRICHMENT OF PHAGES DISPLAYING SINGLE CHAIN ANTIBODIES,** Vijay K. Chaudhary, Smita Abrol and Ira Pastan*, Department of Biochemistry, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India and *Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892.

A phagemid system to produce phage particles displaying single chain antigen binding domains of antiTac, an antibody against the p55 subunit of the human IL2 receptor, fused to the gene III protein of M13 has been developed. Purified phages displaying the single chain antibody were able to bind to soluble biotinylated p55 and this binding was competed by excess of antiTac. These phages also bound selectively to a transfected cell line, ATac4, that express p55 (Tac) subunit of the human IL2 receptor but not to the parent cell line A431, which does not express p55 subunit. This system may be useful for selecting and isolating high affinity antibodies against cell surface molecules by differential screening on cells that express (or do not express) the antigen.

T 306 PRODUCTION OF A HIGH AFFINITY ANTI-CEA scFv FOR COLORECTAL TUMOUR TARGETING.

Chester KA[1], Begent RHJ[1], Robson L[1], Keep PA[1], Pedley RB[1], Boden J[1] Boxer G[1], Green A[1], Winter G[2], Cochet O[2] and Hawkins RE[2]. [1]CRC Laboratories, Department of Clinical Oncology, Royal Free Hospital School of Medicine, London NW3 2PF, UK. [2]MRC Centre, Hills Road, Cambridge CB2 2QH, UK.

Antibodies with desired characteristics may be isolated from libraries of single chain Fv (scFv) in filamentous phage by manipulating the selection conditions (Hawkins *et al.*, J. Mol. Biol. 1992; **226:** 889-896). This was exploited to produce a high affinity antibody to carcinoembryonic antigen (CEA). Mice were immunised with CEA, spleen mRNA extracted, and the variable region genes amplified from cDNA by PCR using specific primers. The PCR products were cloned as scFv (VH and VL joined by a flexible linker) into bacteriophage vectors producing a library of 10^7 members. Antibody with specificity and high affinity for CEA was selected by allowing the phage library to bind biotinylated CEA at low concentrations (5nM). The selected clone was subcloned for expression as a soluble scFv linked to a C-terminal myc tag to aid identification during protein purification. Approximately 20 mg per litre of scFv was obtained from bacterial supernatant. This was purified by CEA-Sepharose affinity chromatography and by size exclusion gel filtration. The dissociation constant (K_d) was shown by fluorescence quench to be 2.5+/- 1.3 nM, indicating the high affinity binding to CEA in comparison to a K_d of 25 nM in the same assay for A5B7, a monoclonal antibody which has produced some of the best colorectal tumour targeting to date. Immunohistochemistry showed a specific CEA-reactive pattern in 10/10 human colorectal adenocarcinomas. Tumour localisation in vivo was studied using LS174T human colorectal tumour xenografts in nude mice. Specific tumour imaging was obtained using ^{125}I-scFv, and localised radioactivity in excised tissues showed tumour:blood ratios of 11:1 at 24 hours after administration of ^{125}I-scFv and 53:1 at 48 hours. This is the first example of a high affinity, high specificity anti-CEA antibody produced by phage technology and our data indicate that it has potential for improved imaging and therapy of colorectal cancer.

T 307 DIVERSITY OF HUMAN ANTIBODY TO THE HAEMOPHILUS INFLUENZAE B CAPSULAR POLYSACCHARIDE DEFINED WITH COMBINATORIAL SURFACE PHAGE EXPRESSION LIBRARIES, Yu-Waye Chu, Mark Sullivan,* Richard Insel. Dept. Pediatrics, Univ. Rochester Sch. Med.; *Clin. Diag. Div., Eastman Kodak Co., Rochester, NY, 14642

The human antibody (Ab) repertoire to the capsular polysaccharide (CP) of the bacteria Haemophilus influenzae (Hib) has been partially defined based on the amino acid sequence of purified Abs or the nucleotide sequence of the immunoglobulin genes of Ab-secreting cell lines. This repertoire is highly restricted with use of a limited number of human V_H genes and preferential use of the Vκ2-A2 gene. To better define this repertoire, combinatorial surface phage expression libraries are being generated starting with RNA isolated from human postimmunization lymphocytes. Combinatorial libraries are also being generated by pairing a specific V_H or V_L from hybridomas that secrete Ab to the Hib CP to the respective V library. The sequences of the isolated clones will be compared to the sequences from purified Abs or Ab-secreting cell lines to determine the ability of in-vitro generated combinatorial phage Ab libraries to replicate the in-vivo Ab repertoire.

T 308 CLONING AND EXPRESSION OF AN ANTI-PDI scFv IN E.COLI, J.R.Clark and R.B.Freedman, Biological Laboratory, University of Kent at Canterbury, Canterbury, Kent, CT2 7NJ, UK

A scFv has been cloned from a hybridoma (RL77) which secretes a mAb directed against PDI (protein disulphide isomerase), and expressed in *E.coli* as a fusion protein with the M13 gene 3 protein using the vector pCANTAB5 (Pharmacia). The scFv construct (pRL77) was subsequently modified to allow soluble scFv expression in an amber suppressing strain by the insertion of an oligonucleotide encoding the FLAG peptide (IBI Ltd.), and an amber codon downstream of the scFv gene. Specificity for PDI by both RL77 phage antibody and soluble scFv has been demonstrated by ELISA and Western blotting. High-level expression of the FLAG-tagged scFv has been achieved (100-200 mg per l) and the tagged scFv has been purified by affinity chromatography. The modified vector will used in the construction of a combinatorial library to be panned against bovine PDI. Soluble scFv antibodies will be used in structural and functional studies of PDI.

Acknowledgement. We are very grateful to Dr Charlotte Kaetzel (Case Western Reserve University, Ohio) for the gift of a panel of anti-PDI mAbs and hybridomas.

T 309 **USE OF FIXED TISSUE SECTIONS OR CELLS IN BIO-PANNING OF F_{ab}-ANTIBODY LIBRARIES.**
Jan Engberg, Morten Dziegiel, Peter S. Andersen, Anne Øster, Leif K. Nielsen and Lene K. Johansen.
The Royal Danish School of Pharmacy, Dept. of Biology, 2 Universitetsparken, DK-2100 Copenhagen and The University Hospital, Dept. of Clinical Immunology (K12031), 9 Blegdamsvej, DK-2100, Copenhagen, Denmark.

We have developed efficient methodologies for construction and expression of comprehensive phage-display libraries of murine and human F_{ab} fragments in *E.coli* cells. Our methods optimize several critical steps of the PCR amplification of transcripts of the re-arranged immunoglobulin genes and their subsequent assembly and expression (cf. Nucl.Acids Res.(1993) **21**,4491-4498).
Using these methods, we have generated libraries from spleens of immuniced mice and from blood samples obtained from patients. The human libraries from patients with high titers of anti-Rhesus D antibodies were screened by a newly developed bio-panning method using formaldehyde-fixed erythocytes isolated from individuals expressing the Rhesus D antigen. The positive clones exhibited exclusive specificity for the RhesusD antigen.
Using immunocytochemical methods and a F_{ab}-phage with specificity to human insulin we have defined conditions to detect beta cells specifically in formalin fixed pancreas sections. We are using this model system to find conditions to perform bio-panning of complete libraries on fixed tissue sections.

T 310 CONSTRUCTION OF A COMBINATORIAL ANTIBODY LIBRARY FROM AN INSULIN-DEPENDENT DIABETIC PATIENT. Alberto Falorni, Mats A.A. Persson, Annemette Borch, Bengt Persson and Åke Lernmark. Departments of Endocrinology, Medicine and Pediatrics, Karolinska Institute, Stockholm S-171 76, Sweden

Insulin-dependent diabetes mellitus is an autoimmune disease characterized by the high occurrence of Glutamic Acid Decarboxylase (GAD65) autoantibodies (GAD65Ab). Using our previously described radioimmunoassay, we selected a GAD65Ab-positive 9-year old female diabetic patient. First-strand cDNA was made from total RNA (16 ug) prepared from peripheral lymphocytes using reverse transcriptase and *Not* I-d(T)18 primer. PCR amplification of the cDNA was carried out using a number of biotynilated primers hybridizing to the 5'end of the V region of heavy (G1) and light (k) chain and 3' biotynilated primers associating to the CL or CH1/hinge regions. The PCR amplified fragments were gel purified, digested with restriction enzymes and purified with streptavidin-bound magnetic beads. Restriction enzymes were *Xba*I and *Sac*I for the k-chain DNA and *Xho*I and *Spe*I for the heavy-chain DNA respectively. Prepared k-chain PCR product (375 ng) was ligated with 2.5 ug of a prepared *Xba*I/*Sac*I pCombIII phagemid, containing the gene III codifing for a 406-residue minor phage coat protein, cpIII. DNA was precipitated, resuspended in 15 ul dH_2O and transformed by electroporation into NM522 cells. After electroporation, cells were incubated in 20 ml Super Broth containing 1% glucose and 20 ug/ml ampicillin at 37°C for 1 hr. Aliquots of 0.5 and 0.05 ml were plated and k-chain library size determined: $4x10^7$ cfu. By PCR amplification, using T3 and T7 primers, the insert frequency was estimated to be 80%. Transformed cells were incubated overnight at 37°C in 200 ml Super Broth containing 1% glucose and 50 ug/ml ampicillin, and the phagemid DNA (containing the k-chain library) prepared and digested with *Xho*I and *Spe*I. The heavy-chain PCR product (210 ng) was then ligated into 1.5 ug of the prepared *Xho*I/*Spe*I phagemid DNA, in fusion with the gene III. The ligated DNA was transformed into NM522 cells and the final heavy/k chain library made following the procedures above described. The size of the combinatorial library was $4x10^6$ and the fraction of successfully ligated phagemids (both heavy and light chain DNA) estimated to be 70%. Selection against recombinant GAD65 in order to isolate GAD65 specific antibodies is in progress.

T 311 PANNING FOR PLATELET SPECIFIC FAB FRAGMENTS BY FACS, Deborah L. French, Jeffery L. Kutok, Barry S. Coller and Christine M. Grimaldi, Departments of Pathology and Medicine, SUNY at Stony Brook, Stony Brook, NY 11794-8691.

The aggregation of platelets at the site of vascular injury is mediated by the platelet GPIIb/IIIa receptor. This calcium dependent heterodimeric receptor is present as a complex on the surface of resting platelets. After agonist activation, high affinity binding sites for fibrinogen become exposed due to a change in the conformation and/or microenvironment of the receptor complex. A murine monoclonal antibody (mAb), 7E3, has high affinity for the activated GPIIb/IIIa complex and inhibits platelet aggregation *in vitro* and *in vivo*. The generation of human mAbs that have the binding characteristics of 7E3 would be useful diagnostic and therapeutic agents. To identify Fab fragments that are expressed on phage particles that recognize the GPIIb/IIIa complex, panning on whole platelets was tested. The heavy and light chain genes from a GPIIIa specific mAb, 7H2, that inhibits platelet aggregation *in vitro* were cloned into the phage display vector, pComb3. Phage particles (10^{11}) were incubated with whole platelets followed by the addition of a biotinylated anti-M13 antibody and streptavidin-FITC. The platelets were analyzed by FACS and a single peak of fluorescense was obtained. Phage particles were eluted with acid, amplified, and reanalyzed by FACS resulting in the same profiles. The specificity of the Fab expressing phage particles for GPIIIa were demonstrated by competitive inhibition with whole 7H2 mAb. Soluble Fab fragments were generated and bound specifically to purified GPIIb/IIIa by ELISA and whole platelets by fluorescense. This methodology will be very useful for identifying Fab fragments that are specific for conformation dependent receptors and will be utilized for screening our human libraries.

T 312 EVALUATION OF HUMAN HEAVY CHAIN IMMUNOGLOBULIN LOCI-DERIVED YACS IN MICE, Larry L. Green, Margaret C. Hardy, Catherine E. Maynard-Curry, Hirohisa Tsuda, Donna M. Louie, Douglas H. Smith, Michael J. Mendez, Sue Klapholz and Aya Jakobovits, Cell Genesys, Inc., 322 Lakeside Drive, Foster City, CA 94404

Production of a diverse repertoire and significant levels of human antibodies in mice will require large segments of DNA containing many V, D and J coding segments, a constant region, and defined and undefined controlling elements. Human immunoglobulin loci cloned in yeast artificial chromosomes (YACs) are a potentially valuable source of immunoglobulin DNA in its native, germline configuration. Using ES cell-yeast spheroplast fusion technology that we have developed, we have introduced large fragments of the human heavy chain locus into the mouse germline. In this poster, we present evaluation of these mice.

T 313 ANALYSIS OF KAPPA CHAIN IMMUNOGLOBULIN LOCI-DERIVED YAC'S IN MICE, Margaret C. Hardy, Larry L. Green, Catherine E. Maynard-Curry, Hirohisa Tsuda, Donna M. Louie, Douglas H. Smith, Hadi Abderrahim, Sue Klapholz and Aya Jakobovits, Cell Genesys, Inc., 322 Lakeside Drive, Foster City, CA 94404

Large fragments of DNA encoding a constant region, many V and J sequences, and known and unknown regulatory elements are thought to be necessary for the production of a broad repertoire of human antibodies by mice. Yeast artificial chromosomes (YAC's) provide a powerful means to clone the human kappa chain locus in a native, germline configuration. We have previously described a method of ES cell-yeast spheroplast fusion that we have used to introduce the human kappa chain locus fragments into the mouse germline. In this poster, we describe the analysis of these mice.

T 314 HUMAN THYROID AUTOANTIBODIES FROM pComb3 PHAGE DISPLAY COMBINATORIAL LIBRARIES.
J. Mark Hexham, Lynda J. Partridge, Jadwiga Furmaniak, Vaughan B. Petersen, Jeremy C. Colls, Chris A.S. Pegg, Bernard Rees Smith and Dennis R. Burton. Molecular Biology, PO Box 594, Sheffield University, Sheffield, S10 2TN, England.

Thyroid lymphocyte RNA from a Hashimoto patient with high serum autoantibodies against thyroid peroxidase (TPO) was used to construct a phage display antibody library in the phagemid vector pComb3. IgG1 and κ antibody genes were amplified by PCR and combined to produce a phage display library (100,000 cfu), expressing Fabs on the surface of filamentous phage particles. This library was then enriched for phage bearing anti-TPO Fabs by consecutive rounds of "panning" against TPO-coated ELISA wells. Plasmid DNA was prepared from the selected population and used to express soluble Fabs which were tested by ELISA for TPO binding. 3 different, novel Fabs specific for TPO were identified with apparent affinities in the region of 10^9 M^{-1}. The Fabs inhibited binding of donor patient's serum to TPO and recognised at least two distinct epitopes on TPO. Sequence analysis revealed a restricted pattern of Vκ gene usage whereas heavy chain gene usage was much more diverse. Available sequence data showed that these same Vκ light chain germline genes are commonly used by other antibodies with both self and non-self specificities. No clear relationship between gene family or germline gene usage by TPO autoantibodies of similar epitope specificity could be identified.

T 315 HUMAN SINGLE-CHAIN IMMUNOGLOBULIN VARIABLE FRAGMENTS (scFvs) TO FACTOR VIII, A MODEL TO STUDY THE V-GENE REPERTOIRE OF AUTOANTIBODIES, Hoet R.M.A.[1)3)], Finnern R.[1)], Kramer M.[2)], G. Kemball-Cook[2)], van Venrooij W.J.[3)] and Ouwehand W.H.[1)2)], [1)]Division of Transfusion Medicine, University of Cambridge, Cambridge CB2 2PT, U.K., [2)]National Institute of Biological Standards and Control, Potters Bar, U.K., [3)]Department of Biochemistry, University of Nijmegen, the Netherlands.

We are establishing patient-related libraries to study the V-gene repertoire in different autoimmune diseases. Factor VIII (FVIII) was used as a model antigen, since autoantibodies as well as alloantibodies to FVIII do occur.

Firstly, the V-gene repertoire from two non-immune healthy individuals (see Marks *et al.*, J.Mol.Biol.(1991) **222**: 581-597) has been investigated for the presence of FVIII antibodies. Phage displaying scFvs against recombinant Factor VIII (rFVIII) were obtained by four rounds of selection using rFVIII as the antigen. Two unique clones showed reactivity with rFVIII in ELISA, 8C8 and 8H2. Specificity of soluble scFv produced by both clones was analysed on a panel of 18 protein and carbohydrate antigens in ELISA. Both clones were reactive with rFVIII and plasma-derived FVIII, 8H2 only reacted with FVIII while 8C8 reacted also with polyclonal IgG and IgM.

The binding of 8H2 scFv was inhibited by the serum of half of the haemophilia patients with inhibiting antibodies to plasma-derived FVIII, and half of the patients with autoantibodies against FVIII. Nucleotide sequencing revealed that VH genes of both clones were derived from the VH1 family (DP10 and DP15) and that the 8C8 clone produced a single 'heavy domain only'antibody fragment (dAb). This VH1-DP10 encoded dAb was recombined with the light chain repertoire derived from two healthy individuals. 4% of these light chain variable domains were able to support binding to rFVIII.

Currently we are establishing a patient-related V-gene phage display library from a haemophilia patient to investigate the V-gene derivation and level of somatic mutation of immune FVIII antibodies. Therefore, B-cells against rFVIII were selected, using binding to rFVIII coated plates. These antigen selected B-cells were used to make a random recombinatorial V-gene library which is now being selected for FVIII binders.

The B-cell selection method developed for FVIII will be used to study the V-gene autoantibody repertoire to other autoantigens, particulary, autoantibodies to U1 RNA in systemic lupus erythematosus (SLE).

T 316 HUMAN ANTIBODIES DERIVED FROM RODENT ANTIBODIES VIA EPITOPE IMPRINTED SELECTION (EIS). Hennie R. Hoogenboom, Laurent Jespers, Steven Mahler, Andy J. Roberts, David J. Chiswell and Greg Winter; Cambridge Antibody Technology Ltd, The Science Park, Unit B1, Melbourn SG8 6EJ, Cambridgeshire, UK.

We demonstrate a new method, termed 'Epitope imprinted selection' (EIS), based on chain shuffling and selection, for converting animal antibodies with defined antigen binding activities into completely human antibodies. The V-genes from a rodent antibody are shuffled with repertoires of human partner genes, and binding combinations selected on antigen by phage display. The rodent V-domain helps to 'dock' the human partner V-domain onto the same epitope. Sequential shuffling or combining selected domains from parallel shuffling can then generate completely human antibodies.

Using this approach we have converted several murine antibodies, including a murine anti-TNF antibody. For example, from the latter antibody, the V_H and V_κ genes were isolated and used as docking chains for human partner chain repertoires. After various chain shuffling and selections experiments, several human V-gene combinations were isolated. One of the human antibodies was recloned and transfected into SP2/0 cells to be produced as a complete IgG1/λ human antibody. The antibody competes with the original antibody for binding to antigen, and has a similar affinity. Thus, the combination of domain shuffling and selection on antigen using the original rodent V-genes as 'docking' chains, yields entirely human antibodies imprinted with the same binding specificity.

T 317 EXPRESSION OF HUMAN HEAVY CHAIN IMMUNOGLOBULIN GENE YACs IN TRANSGENIC MICE, Dennis Huszar, Paul Hollenbach, Donna Munoz-O'Regan, Ted Choi, Condie E. Carmack. GenPharm International Inc., 297 North Bernardo Avenue, Mountain View, CA 94043

We (Choi et al. Nature Genetics, 1993, 4: 117-123) have introduced an 85 kb fragment of the human immunoglobulin heavy (H) chain locus, cloned in a yeast artificial chromosome (YAC), into the mouse germline by co-lipofection into ES cells with an unlinked marker plasmid . The J1.3 YAC insert comprises a Spe I restriction fragment encoding V_H6, all of the functional D segments, all six J_H segments and the Cμ constant region. J1.3 transgenics were bred with Ig H chain-deficient "J_HD" mice, generated by targeted deletion of J_H gene segments (Chen et al, Int. Immunol. 1993, 5: 647-656), to produce mice capable of producing exclusively human μ chains. The J1.3/J_HD transgenics expressed low serum levels of human μ, but were capable of mounting an antibody response to the T-dependent antigen KLH-DNP. Sequence analysis of CDR3 junctions from the spleens of naive transgenics identified the presence of multiple unique clones demonstrating a diversity of J1.3 DJ rearrangements.

The J1.3 transgene encodes a limited primary repertoire; the generation and analysis of transgenics bearing larger YAC Ig H chain clones containing additional V_H and C_H genes is underway.

T 318 IMPROVED VECTORS AND HOST STRAINS FOR EXPRESSION OF FAB ANTIBODY FRAGMENTS ON PHAGE. Johansen, L. K., Albrechtsen B., Øster, A. and Engberg, J., Department of Biology, The Royal Danish School of Pharmacy, Universitetsparken 2, DK 2100 Copenhagen.

We have constructed an improved phagemid vector that contains a bi-cistronic transcription unit for two-step cloning and expression of murine Fab antibody fragments in *E. coli*. The inherent problems with PCR assembly of the heavy and light-chain genes (low yields, artefacts etc.) can be avoided by the use of this vector. Additionally, the vector contains the murine $CH1_{\gamma 1}$ gene fragment fused to the truncated geneIII (ΔgIII) from the filamentous phage, F1. Therefore it is possible to PCR amplify the Fv-heavy chain gene fragments from the complete Ig-repertoire using only four J-region primers and 25 heavy variable region primers. The amplified Fv-heavy and light chain genes are subsequently equipped with restriction sites in a secondary PCR reaction and cloned in separate steps.

Several additional features of the vector should be noted: A sequence coding for a trypsin cleavage site is present at the junction between the $CH1_{\gamma 1}$ gene and ΔgIII. This facilitates gentle and efficient elution of Fab-phage independent of their binding strength and binding type during the panning procedures. ΔgIII can easily be removed since it is bracketed by EagI sites. Its removal fuses the heavy chain gene fragment to a downstream $(His)_6$ tag sequence, which facilitates purification of the resulting soluble Fabs. Truncation of gIIIp reduces host-cell resistance to superinfection. Furthermore, to minimize the toxicity from ΔgIIIp and the antibody fragment the wild type *lac* promoter is used in the transcription unit, so that expression is repressed unless the inducer, IPTG, is added. A similar vector for cloning of human Fab antibody fragments has also been made in the laboratory.

Finally, to optimize the system we have expressed Fab-fragments in different host strains and found a significant difference in the strains' ability to make functional Fab-fragments.

T 319 THE ISOLATION OF NUCLEOPROTEIN COMPLEX COMPONENT-BINDING ANTIBODY FRAGMENTS FROM AUTOIMMUNE HUMAN-DERIVED BACTERIOPHAGE DISPLAY LIBRARIES, Marie T. Kremer and Susan L. Deutscher, Department of Biochemistry, University of Missouri, Columbia, MO 65212

Display of antibody fragments (Fab) on the surface of filamentous bacteriophage and selection of phage that bind to a particular antigen has enabled the isolation of Fab with numerous specificities. We have examined the possibility of isolating nucleoprotein complex-binding Fab by constructing a combinatorial library of phage displaying Fab derived from the antibody repertoire of autoimmune humans. Patients selected exhibit mixed connective tissue disease (MCTD), which is a subset of systemic lupus erythematosus. MCTD patients contain antibodies reactive against various nucleic acid, protein, and nucleoprotein complexes, most notably those involved in RNA processing. The cDNA library was constructed from total RNA derived from leukophoresed patients and the resulting Fab genes were expressed in *E.coli* using the pComb system[1]. This library was panned against various DNA and RNA molecules. Several positive clones were then isolated from the reactive colonies. DNA sequence analysis has shown that the Fab clones contain unique heavy and light chains. The expressed Fabs will be further analyzed for binding characteristics.

[1]Barbas, C.F. III and Lerner, R.A. (1991) Methods: Comp. to Methods Enzymology 2, 119-124.

T 320 POTENTIAL PITFALLS IN THE MICRO-BIO-PANNING SELECTION OF ANTIBODIES DISPLAYED ON FILAMENTOUS PHAGES, Kretzschmar, T. and Geiser, M., K-681.5.10, Biotechnology, Ciba-Geigy Ltd., 4002 Basel, Switzerland.

Random libraries of functional antibody fragments can be expressed on the surface of filamentous bacteriophages. Those phages displaying an antibody with a defined specificity can be selected by the so-called "micro-biopanning" technique (Parmley and Smith, 1988): Phage libraries are incubated with antigens fixed in wells of a microtiter-plate. Specifically bound phages are regained by, e.g., washing the wells at a low pH or applying an excess of soluble antigen.

We will present data showing that this kind of selection may explain failures in the identification of interesting phages. Especially, when libraries derived from a non-immunized organism are constructed and rare phage species with a rather low affinity should be isolated, the limits of micro-biopanning are becoming manifest.

We compare the efficacy of this micro-biopanning to a selection by column affinity chromatography, and discuss in detail the parameters which are essentiell for isolation of relevant recombinant phages.

Parmley, S.F. and Smith, G.P. (1988) Gene 73, 305.

T 321 HUMAN SCFV ANTIBODIES FROM SEMI-SYNTHETIC PHAGE DISPLAY LIBRARIES, T. Logtenberg, E. Boel* and J. de Kruif, Departments of Immunology and *Microbiology, University Hospital Utrecht, Utrecht, The Netherlands.

Current efforts in antibody engineering are aimed at generating 'master' libraries of antibodies that contain all conceivable specificities. We have constructed a semi-synthetic phage antibody library by linking 49 human germline VH gene segments from all 6 families (representing different canonical sequences) to randomized CDR3 regions varying in length from 6 to 15 amino acid residues. These VH regions were coupled to 6 different germline κ and λ V regions and expressed as geneIII fusion proteins on phage using the pHen1 vector. This resulted in the generation of a library containg $> 5 \times 10^8$ specificities. The quality of the library was tested against a variety of antigens including proteins, peptides, haptens and polysaccharides. Some of the antigens in the panel are highly conserved throughout evolution and were selected because we failed to generate conventional murine monoclonal antibodies by somatic hybridization. We report on the succeful isolation and characterization of human scFv antibodies against these antigens.

T 322 PREPARATION OF MONOCLONAL ANTIBODY Fabs AGAINST RICIN BY RECOMBINANT DNA TECHNIQUES, John L. Middlebrook, Theresa Smith and Paul Lemley, Toxinology Division, U. S. Army Medical Research Institute of Infectious Diseases, Frederick, Md 21702

Ricin is a highly toxic plant protein that acts by inhibiting eucaryotic cell protein synthesis. We attempted to develop neutralizing monoclonal antibodies against ricin by recombinant techniques. A mouse was immunized against the toxin by use of a toxoid, followed by several challenges with native toxin. Total RNA from spleen cells was used to prepare cDNA immunoglobulin light and truncated heavy chain libraries. A random recombinatorial library was then prepared using modified lamdaZAP vectors and screened for radiolabeled ricin binding. Twenty-two of approximately 25,000 clones examined exhibited ricin binding activity and these were isolated for further study. All of the clones appeared to express only light or truncated heavy chain fragments, but not both. Several of the truncated heavy chain expressing clones were "chain shuffled" with the original light chain library in an attempt to obtain ricin binding Fab fragments. Two clones were isolated that produced material expressing properties of a Fab when analyzed by western blotting and BIAcore analysis. Both clones, however, produced very low amounts of the Fab and further characterization of the material was difficult.

T 323 IN VITRO MIMICKING OF ANTIBODY GENE SOMATIC HYPERMUTATION BY A LIGASE CHAIN REACTION STRATEGY, Saran A. Narang, Su-jun Deng, C. Roger MacKenzie, David R. Bundle and N. Martin Young, Institute for Biological Sciences, National Research Council of Canada, Ottawa, Canada, K1A 0R6

Simultaneous randomization of antibody CDRs resembles the somatic hypermutation stage of the immune response. We have developed a synthetic ligase chain reaction strategy for the controlled and simultaneous randomization of all V_H CDRs during gene assembly. A mathematical analysis indicated that spiking the CDR nucleotides at a level of 10% introduced amino acid substitutions to a degree approximating that observed in the late primary and subsequent phases of the immune response. The strategy was tested with a gene library encoding single-chain Fv specific for the Salmonella serogroup B O-polysaccharide. Mutants with higher antigen-binding activity, relative to the wild-type, were isolated by phage display. Several mutational hot spots were associated with improved binding but none involved residues that contact antigen in the crystal structure, indicating that structural changes exert an indirect effect on ligand binding. It is suggested that CDR randomization, followed by the selective pressure of phage display, may find general application as a means of mimicking the affinity maturation process in vitro.

T 324 ANTIBODY FRAGMENTS FROM A "SINGLE POT" PHAGE DISPLAY LIBRARY AS IMMUNOCHEMICAL REAGENTS, Ahuva Nissim, Hennie R. Hoogenboom, Ian M. Tomlinson, Greg Flynn, Carol Midgley, David Lane and Greg Winter, MRC Centre for Protein Engineering, Hills Road, Cambridge. CB2 2QH. U. K

The display of repertoires of antibody fragments on the surface of filamentous bacteriophage offers a new way of making antibodies with predefined binding specificities. Here we explored the use of this technology to make immunochemical reagents to a range of antigens by selection from a repertoire of > 10^8 clones made *in vitro* from human V-gene segments. From the same "single pot" repertoire, phage were isolated with binding activities to each of 18 antigens, including the intracellular proteins p53, elongation factor EF-1α, immunoglobulin binding protein, rhombotin-2 oncogene protein and sex determining region Y protein. Both phage and scFv fragments secreted from infected bacteria were used as monoclonal and polyclonal reagents in Western blots. Furthermore the monoclonal reagents were used for epitope mapping (a new epitope of p53 was identified) and for staining of cells. This shows that antibody reagents for research can be readily derived from "single pot" phage display libraries.

T 325 HUMAN AND MACAQUE Fab MOLECULES SPECIFIC FOR IMMUNODEFICIENCY VIRUSES ISOLATED BY PHAGE DISPLAY TECHNIQUES. Mats A.A. Persson*, Jorma Hinkula***, Astrid Samuelsson**, Karin Lundin*, Britta Wahren***, Erling Norrby**, Francesca Chiodi**. Karolinska Institute, Department of Medicine*, Karolinska Hospital, S-171 76 Stockholm; Karolinska Institute, Departments of Virology** and Disese Control***, c/o SMI, S-105 21 Stockholm; Sweden

In a first set of experiments, we generated a combinatorial γ1/κ Fab library on phage from bone marrow lymphocytes of an HIV-1 infected, asymptomatic Swedish individual, using the pComb3 vector. Three rounds of selection for phage binding to HIV-1 envelope protein $gp120_{LAI}$ resulted in a 250-1000 fold enrichment of specific phage. We have analysed 21 antigen specific Fab clones from this library in depth. They comprise a diverse set of high-affinity antibodies with different bio-functional characteristics. Eleven neutralize HIV-1 virus *in vitro* in a cross strain manner: though selected by using gp120 of the LAI strain, a majority of our cloned neutralize $HIV\text{-}1_{MN}$ more efficiently than $HIV\text{-}1_{LAI}$ (neutralixation defined as >50% reduction in p24 production). When testing two of the neutralizing Fab clones for neutralization *in vitro* of primary HIV-1 isolates from donors in Sweden and Uganda, the two Fab:s together neutralize eight of ten European isolates *in vitro* at a concentration of 1 µg/ml or less.

Using the same Fab library, we subsequently selected phage clones that bound to a mixture of three overlapping peptides covering a conserved, linear epitope with putative importance for neutralization localized to the second HIV-1 envelope protein gp 41 (aa 657 - 681). We have restricted our inital analysis to some 25 different clones. Using an assay where binding to gp160 on solid phase were inhibited by peptide in solution, we identified ten clones clearly specific for one of the three peptides used, and thus reactive distinctly to a linear epitope on gp41. Their heavy chains are unrelated. Six of these neutralize HIV-1 strains SF2, MN, and LAI, and 3 - 4 out of 5 primary isolates from Swedish donors.

Preliminary experiments using combinations of anti-gp41 and gp-120 specific Fab:s have given us data indicating synergy when neutralizing HIV-1 *in vitro*. Thus, combinations of antibodies may be more efficient for immuno-therapy than monoclonal antibodies given separately. To study this, and other optimization of passive immunotherapy in an appropriate animal model, we have recently been able to isolate SIV gp110Sm specific Fab fragments from a γ1/λ library established from peripheral blood of an SIVSm infected, but still healthy, cynomologus monkey. Initial analysis of 14 SIV specific Fab clones showed them to be clonally unrelated, and cross strain reactive (SIVmac) to different degrees. Their bio-functional properties are currently being analysed.

T 326 DEVELOPMENT OF A NOVEL SOFTWARE TOOL FOR IMMUNOGLOBULIN SEQUENCE ANALYSIS: SEQUENCE ANALYSIS WORKSHOP FOR WINDOWS. Harry W. Schroeder, Jr. and Rotem A. Elgavish, Division of Developmental and Clinical Immunology, Departments of Medicine and Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294-3300

Investigators seeking to glean information from a novel human antibody sequence must first identify the likely germline VDJ progenitors from more than 100 VH, 30 DH and 6 JH gene segments for the H chain and from a slightly lesser number of VL and JL gene segments for the L chain. Only then can the identity and consequences of somatic changes, if present, be determined. In the past, this process has primarily been done by hand, or laboriously on a sequence by sequence basis on the computer. In order to simplify the process of sequence analysis, we have written a Windows™ based computer program called SAW (for Sequence Analysis Workshop) designed to provide rapid, real-time comparisons of antibody variable domains. Sequences for SAW can be obtained from Genbank or from a standard text file. SAW can compare a variable domain to as many as 100 different sequences, calculate the extent of homology, sort the sequences by their degree of similarity, translate the nucleotide codons into amino acids, and then display the results in either a graphical or text format. These comparisons allow the investigator to determine the likely germline progenitors of a variable domain and to visualize how it differs from other antibody genes by functional region. SAW supports replacement and silent site substitution analysis by either codon or region, thus providing rapid insight into the forces that have shaped mutations. The sequence comparisons can be printed out as an aid for paper analysis or for preparation of figures for publication. SAW was written in Visual C++ 1.0 as an application for Microsoft® Windows™. Windows™ applications allow the investigator to see results in several windows at once, each of which can be independently manipulated. SAW uses four subsidiary windows that can be manipulated by the investigator, as well as several other analysis windows that can be opened and closed at will. The use of color and graphics, the generation of subsidiary windows that contain the results of specific analyses, and mouse-driven control of the program make SAW an easy to use tool for immunoglobulin sequence comparison.

T 327 DH READING FRAME PREFERENCE IN MAN. IMPLICATIONS FOR SELECTION OF THE B CELL REPERTOIRE. Satoshi Shiokawa, Frank Mortari, César Nuñez, and Harry W. Schroeder, Jr., Division of Developmental and Clinical Immunology, Departments of Medicine and Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294-3300

The CDR 3 domain of the heavy chain helps create the center of the antigen binding site, hence its sequence and structure can prove critical to antibody specificity. CDR 3 sequence diversity is the product of VDJ joining and N region addition. Variation is enhanced by the potential of each DH gene segment to be read in six different reading frames, three by deletion and three by inversion. In the mouse, preference for only one of these reading frames is the result of the elimination of reading frames that encode termination codons or a translatable Dμ protein, preferential splicing at sites of sequence identity between the rearranging gene segments, and a preference for deletion over inversion. The latter is of particular interest because murine pathologic antibodies often exhibit charged CDR 3 regions that can only arise by inversion. We examined the sequences of fetal DJ and VDJ transcripts containing DHQ52, which is used in up to 40% of fetal transcripts in man. Human DHQ52 typically undergoes deletional joining. None of the reading frames in deletional orientation have open upstream reading frames. One of the three reading frames is neutral in polarity, one is hydrophilic, and the last reading frame contains a termination codon. More than 90% of DHQ52 joins in both DJ and VDJ joins from first trimester and second trimester tissues contained N regions, hence sequence homologies could not play a role in reading frame preference; and indeed DJ transcripts did not show a preference. VDJ transcripts from pre-B cells demonstrated selection against the deletional reading frame encoding the termination codon, yielding rearrangements with a hydrophilic and a neutral CDR 3. However, VDJ transcripts from unselected B cells exhibited preference only for the neutral reading frame, suggesting selection based on the charge characteristics of the CDR 3 domain. Examination of the DJ and VDJ joins using other DH gene segments exhibited a similar preference for a neutral CDR 3. In man, B cells undergoing VDJ rearrangement appear to undergo selection against H chains exhibiting highly polar or hydrophobic antibody binding sites. This selection process may play a major role in the development of B cell tolerance. We performed a similar hydrophobicity analysis on murine DH gene segments, and found the preferred reading frame contained a similarly limited characteristic charge distribution. The preferred human DH hydrophobicity index is slightly more positive than the one used by mouse, thus each species could potentially have a slightly different set point. If there is indeed a narrow range of selection for a preferred charge characteristic in CDR 3, and this set point does differ, then human antibodies generated in transgenic mice may not be totally accepted as human.

T 328 EXPRESSION AND CHARACTERIZATION OF RECOMBINANT ANTI-RH(D) ANTIBODIES ON FILAMENTOUS PHAGE: A MODEL SYSTEM FOR ISOLATING HUMAN RED CELL ANTIBODIES BY REPERTOIRE CLONING, Don L. Siegel and Les E. Silberstein, Department of Pathology & Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19140

The production of human anti-red blood cell (RBC) immunoglobulins (Ig) *in vitro* from immunized individuals would greatly facilitate the genetic analysis of the human immune response to RBC antigens and also provide useful serological reagents. Technical difficulties inherent in human B-cell immortilization have led to the development of molecular approaches that by-pass the need for cell transformation. By cloning human Ig gene segments into bacterial expression vectors, libraries are created of filamentous phage particles displaying Fab fragments on their surfaces. Libraries have been screened with purified, soluble antigen and selected clones genetically manipulated in *E. coli* to produce soluble Fab fragments. Our goal has been to adapt this technique to the study of RBC auto- and alloantibodies which have specificities against unpurifiable membrane-bound antigens. To test the feasibility of this approach, two sets of phage were created, one set expressing a human anti-Rh(D) Ig and the other expressing a human anti-tetanus toxoid Ig. After verifying the presence of functional phage-displayed Fabs through biochemical, flow cytometric, and electron microscopic analyses, a model library was constructed comprising one anti-Rh(D)-expressing phage per 10^4 anti-tetanus toxoid-expressing phage. A method was developed for screening the library with intact Rh(D)-positive RBCs. After four rounds of panning, anti-Rh(D) specificity was enriched >10,000-fold to a final frequency ~100%. Plasmid DNA derived from anti-Rh(D) phage was used to produce milligram quantities of soluble recombinant anti-Rh(D) Fabs purified by nitrogen cavitation and nickel-chelation affinity chromatography. The authenticity of the Fabs was confirmed by SDS-PAGE and immunoblotting which showed bands with M_r ~50 kD and ~26 kD under non-reducing and reducing conditions, respectively. Binding of recombinant anti-Rh(D) Fabs to Rh(D)-positive RBCs was demonstrated by flow cytometry and by an agglutination assay. Our results suggest that repertoire cloning by cell-surface enrichment may have broad application to the study of the human immune response to erythroid antigens in addition to membrane-bound antigens present on other hematopoietic cells.

T 329 MOLECULAR SELECTION OF HUMAN ANTIBODIES WITH A BACTERIAL Fab-BINDING PROTEIN

Gregg J. Silverman*, Dennis R. Burton† and Minoru Sasano*,*University of California, San Diego, La Jolla CA 92093, †The Scripps Research Institute, La Jolla CA 92037

The bacterial membrane protein, *Staphylococcal protein A* (SpA), has sites that interact with the Fab of many human IgM, IgA, IgG and IgE, and in recent reports we have provided evidence of V_H restriction in Fab that bind SpA. To investigate the molecular basis for this Fab binding specificity, we have used a phage-display combinatorial immunoglobulin library made in the pCOMB3 vector. In the unselected human polyclonal IgG Fab library, about 17% of antibody-expressing clones were found to bind SpA. SpA binding was completely restricted to Fab with V_H3 H chains, and about 60% of V_H3 Fab in the unselected library had SpA binding capacity. Fab were also selected from the library after sequential rounds of panning with SpA. Analysis of 21 V_H sequences and 6 V_L sequences demonstrated that Fab that bind SpA employ diverse V_H3 genes, while the L chains derive from a variety of V_κ and V_λ gene families. By creation of antibodies with differential H-L chain pairing, the global capacity to bind SpA was shown to be dictated by V_H3 usage, but different L chain usage could result in up to a 4 fold change in binding affinity. The apparent K_D of the SpA binding by different Fab ranged from 2.5×10^{-7} to $>10^{-5}$ M. As expected, repeated rounds of panning selected for antibodies based on higher binding affinity. These data indicate that there is a hierarchy within the binding affinities of V_H3 Fab based on V gene usage, and the pattern of V_H3 family restriction of Ig reactive with SpA is comparable to known superantigens for T cells. These data also suggest that certain V_H third framework residues play a critical role in Fab binding of SpA, but binding also appears to influenced by the CDR composition.

T 330 RECOMBINANT ANTI-CEA SINGLE CHAIN FRAGMENTS AND FABS FROM PHAGE LIBRARIES: RAPID DETECTION BY ELECTROCHEMILUMINESCENCE IMMUNOASSAYS

Rodger Smith, Ralph Abraham, Sarah Buxbaum, Scott Kadey, John Link, Michael Darsley, Department of Molecular Engineering, IGEN, Inc., Rockville, MD 20852

A variety of recombinant carcinoembryonic antigen (CEA)-binding fragments were obtained by cloning from three hybridoma cell lines producing anti-CEA monoclonal antibodies. The variable regions of the antibody heavy and light chains or the complete light chains and Fd fragments of the heavy chains were amplified by PCR and introduced into the pCANTAB5 phagemid vector as single chain or Fab constructs, respectively, and were successfully expressed in E. coli. Soluble sFv and Fab fragments were detected in the supernatant of bacterial cultures following induction with IPTG. A new homogeneous immunoassay format based on electrochemiluminescence (Origen® system) was used to detect binding of the proteins to biotinylated CEA. Goat anti-mouse Fab polyclonal $F(ab')_2$ fragments labeled with the electrochemiluminescent ruthenium trisbipyridyl complex were used to quantify anti-CEA Fab fragments using an Origen® Immunoassay Analyzer (IGEN, Inc.). It was possible to measure nanogram amounts of functional antibody fragments as early as three hours after IPTG induction of gene expression in bacterial cultures in a 30 minute assay.

This assay format offers substantial advantages in terms of both speed and sensitivity when compared to more conventional ELISA methods and will be of great value in the screening of heterogenous populations of antibody clones produced, for example, by phage display techniques.

T 331 *Abstract Withdrawn*

T 332 THE COMPLETE MAP OF THE HUMAN V_H LOCUS: SEQUENCE AND STRUCTURAL DIVERSITY OF V_H GENES.

Ian M. Tomlinson, Graham P. Cook, Gerald Walter, Terry H. Rabbitts and Greg Winter, MRC Centre for Protein Engineering/MRC Laboratory of Molecular Biology, Cambridge, CB2 2QH. U. K.

The human immunoglobulin heavy chain locus has been mapped to 14q32.3 by *in situ* hybridisation and 64 V_H segments have been located within 800 kilobase of the J_H segments (Matsuda *et al., Nature Genetics* 3, 88-94).

A 200 kilobase yeast artificial chromosome (YAC) which maps to the 14q telomere was isolated by its ability to confer telomere activity in yeast. This YAC contains 19 V_H segments and completes the map of the human V_H locus. The completed map contains approximately 87 V_H segments (depending on the haplotype), 45 of which have been seen in productive VDJ rearrangements. At least 16 other V_H segments are located on chromosomes 15 and 16 and appear non-functional. Fluorescent *in situ* hybridisation with cosmid and YAC clones reveals two clusters of "orphon" V_H segments on 15q11.2 and 16p11.2. A minor D segment cluster is also located on 15q11.2.

Germline diversity for antigen binding is therefore limited to approximately 45 functional segments which encode seven major loop structures. The germline segments have been cloned as building blocks for use in phage display libraries, allowing the construction of an artificial human immune system *in vitro*.

T 333 INCLUSION OF THE HUMAN IG KAPPA 3' ENHANCER IN A HUMAN KAPPA MINILOCUS CONFERS DOMINANCE OVER THE ENDOGENOUS LAMBDA LOCUS, Mary Trounstine, Lisa D. Taylor, Kay M. Higgins, Stephen R. Schramm, Condie E. Carmack, Dennis Huszar, Nils Lonberg. GenPharm International Inc., 297 North Bernardo Avenue, Mountain View, CA 94043

Transcription of the Ig kappa light chain locus is regulated by the V gene promoter and the Jκ-Cκ intron enhancer, as well as by an additional, recently identified, enhancer situated downstream of the Cκ gene. We have generated transgenic mice containing kappa light chain minilocus transgenes comprised of human Vκ, Jκ and Cκ coding sequences in an unrearranged, germ-line configuration. Minilocus constructs including the intron enhancer, but lacking the downstream enhancer, have been described by Taylor et al. (N.A.R., 1992, 20:6287-6295). To assess the effect of the 3' kappa enhancer, the human sequence was isolated and introduced into the minilocus. Transgenic mice were generated and bred with gene targeted kappa-deficient mice (Chen et al. EMBO J., 1992, 12: 821-830) to produce transgenics capable of producing only human kappa light chains. In mice containing comparable copy numbers of the minilocus constructs, a dramatic enhancement of kappa expression was observed in the presence of the 3' enhancer. The latter mice showed an order of magnitude increase in serum levels of human kappa light chains and a corresponding increase in the intensity of kappa cell surface staining, relative to constructs lacking the downstream enhancer. Furthermore, mice containing low copy numbers of the kappa construct lacking the 3' enhancer could not compete as effectively with the endogenous murine lambda locus, resulting in the production of a high percentage of B cells expressing mouse lambda light chains. In contrast, transgenics with low copy numbers of the 3' enhancer-containing minilocus could efficiently mediate isotypic exclusion, giving rise to a majority of B cells expressing human kappa light chains.

T 334 ISOLATION AND CHARACTERIZATION OF HUMAN Fabs SPECIFIC TO THE RSV MAJOR SURFACE F PROTEIN Tsui, P.; Granger, L.; Tornetta, M.; Ames, R.; Jonak, Z.; Ganguly, S.; Silverman, C.; Porter, T.; Jones, C ;Demuth, S ;and Sweet, R. Departments of Molecular Genetics, Cellular Biochemistry, Protein Biochemistry and Molecular Virology and Host Defense. SmithKline Beecham Pharmaceuticals. King of Prussia, PA 19406.

Antibodies have shown efficacy in animal models of several infectious diseases and also in some clinical settings. The derivation of human monoclonal against infectious agents has been tedious because the murine hybridoma technology has not been readily transferable to the human system. Combinatorial cloning of the expressed human antibody repertoire coupled with phage display has provided a new avenue that has yielded human Fabs against many viral agents. By this methodology, we have isolated a panel of human Fabs specific to the F-protein of the human respiratory syncytial virus (RSV). Libraries prepared from PBL and spleen were selected for binding to recombinant F-protein produced in drosophila cells. Selected clones were initially expressed characterized as Fabs in E. coli and subsequently engineered for production as full-length mAbs in mammalian cells. We will describe the isolation and production of these antibodies and their anti-viral activity in cell culture.

T 335 THE CLONING OF HUMAN GERMLINE IMMUNOGLOBULIN Vλ GENE SEGMENTS FOR USE IN SYNTHETIC ANTIBODY LIBRARIES, Samuel C. Williams and Greg Winter, MRC Centre For Protein Engineering, Hills Road, Cambridge CB2 2QH, UK.

The polymerase chain reaction (PCR) was used to clone 21 human variable gene segments of λ light chains from a single individual. The segments included 10 new $V\lambda$ segments and the representative of a new $V\lambda$ sub-family ($V\lambda$ IX). The segments have been assembled into a repertoire of synthetically rearranged light chain genes. The repertoire reflects the naturally occurring pattern of rearrangement between V, J (joining) and C (constant) segments and incorporates an equivalent degree of diversity in the third antigen-binding loop formed from V-J recombination. The use of these genes in phage-display antibody libraries will be discussed.

Analysis of MAb Structure/Function via Genetic Manipulation

T 400 RECOMBINANT ANTI-CEA SINGLE CHAIN FRAGMENTS AND FABS FROM PHAGE LIBRARIES : RAPID DETECTION BY ELECTROCHEMILUMINESCENCE IMMUNOASSAYS

Rodger Smith, Ralph Abraham, Sarah Buxbaum, Scott Kadey, John Link, Michael Darsley, Department of Molecular Engineering, IGEN, Inc., Rockville, MD 20852

A variety of recombinant carcinoembryonic antigen (CEA)-binding fragments were obtained by cloning from three hybridoma cell lines producing anti-CEA monoclonal antibodies. The variable regions of the antibody heavy and light chains or the complete light chains and Fd fragments of the heavy chains were amplified by PCR and introduced into the pCANTAB5 phagemid vector as single chain or Fab constructs, respectively, and were successfully expressed in E. coli. Soluble sFv and Fab fragments were detected in the supernatant of bacterial cultures following induction with IPTG. A new homogeneous immunoassay format based on electrochemiluminescence (Origen® system) was used to detect binding of the proteins to biotinylated CEA. Goat anti-mouse Fab polyclonal $F(ab')_2$ fragments labeled with the electrochemiluminescent ruthenium trisbipyridyl complex were used to quantify anti-CEA Fab fragments using an Origen® Immunoassay Analyzer (IGEN, Inc.). It was possible to measure nanogram amounts of functional antibody fragments as early as three hours after IPTG induction of gene expression in bacterial cultures in a 30 minute assay.

This assay format offers substantial advantages in terms of both speed and sensitivity when compared to more conventional ELISA methods and will be of great value in the screening of heterogenous populations of antibody clones produced, for example, by phage display techniques.

T 401 COMPLEMENT LYSIS MEDIATED BY ENGINEERED MULTIPLE-DOMAIN FORMS OF THE THERAPEUTIC ANTIBODY CAMPATH-1H.

Judith Greenwood, Scott D. Gorman, Edward G. Routledge, Irene S. Lloyd and Herman Waldmann, Department of Pathology, University of Cambridge, Cambridge, U.K.

Various studies have implicated the CH2 domain of antibodies as the most important region involved in complement activation. It is also known that antigens are more sensitive for complement lysis when present at high density or in patches. We designed a set of antibodies with a view to increasing the number of effector molecules recruited to a single site of antibody-antigen interaction. It was hoped that these might increase the number of target antigens capable of harnessing complement lysis with therapeutic antibodies.
The engineered antibodies all had specificity for the antigen CAMPATH-1 (CDw52), an important therapeutic target found on monocytes and lymphocytes, with extraordinary sensitivity for complement-mediated lysis. Two different types of antibody were produced, one type had domains duplicated intramolecularly, in a tandem arrangement, i.e. two CH2 domains, a hinge-CH2 domain repeat and two whole Fc regions; the second type employed a mutation in which Ser at position 444 is replaced by Cys, allowing the production of antibody dimers.
The ability of these antibodies to direct complement-mediated lysis was tested, and the use of both high and low density antigen targets revealed important changes in antibody potency.

T 402 DESIGN, EXPRESSION, 3D-STRUCTURE AND AFFINITY MATURATION OF BIFUNCTIONAL SINGLE CHAIN ANTIBODIES, Peter J. Hudson[1], Robyn Malby[2], Maria Lah[1], Olan Dolezal[1], Alex Kortt[1], Robert A. Irving[1], and Peter M. Colman[2], CSIRO Division of Biomolecular Engineering[1] and Bio-molecular Research Institute [2], 343 Royal Parade, Parkville, Vic, 3052. Australia
We devised a high-level bacterial expression system (pPOW) for the production of single chain antibodies and other Ig-like molecules. Binding studies on soluble NC10-scFv using ELISA, sedimentation equilibrium and Biacore techniques demonstrated that the scFv had retained similar affinity for NA as the parent Fab to the target antigen, influenza neuraminidase (NA). Over 50mgs of soluble NC10-scFv was produced for crystallization in complex with NA, followed by three-dimensional structural determination by X-ray diffraction. We believe this is the first successful crystallization and structural analysis of an scFv molecule. Preliminary comparisons of the NC10 scFv-NA structure to the NC10 Fab-NA structure indicate that the modes of attachment of Fab and scFv to NA are very similar. Higher resolution analyses will determine the effect (if any) of the peptide linker and peptide affinity tail upon the binding conformation of the scFv. Analysis of the NA-scFv structure revealed a potential dimerisation of scFvs to crosslink NA molecules. This was confirmed by solution studies of isolated scFvs as monomers and dimers and by EM analysis of NA dimer complexes. Molecules were produced with shortened linker polypeptides which forced the scFvs into the dimeric structure. A detailed structural comparison of the NC10 scFv mutants, incorporating either point or random mutations of contact residues, will enable a rational design to affinity maturation.
NC10 scFv were also used to develop an alternative strategy for affinity maturation by mutation and selection from phage-display libraries. We designed a phagemid vector, pHFA for high level expression of antibody fragments (either Fabs or scFvs) that can be produced as: i) soluble molecules incorporating a stable carboxyl-terminal octapeptide (FLAG) or ii) attached to the surface of the Fd bacteriophage gene III protein. Coexpression with DNAK or GroEL/ES charperonins did not improve phage production, but instead resulted in specific proteolysis of the scFv-geneIII fusion proteins. We applied a high-rate mutagenesis strategy using mutD E.coli hosts and selected on the basis of antigen affinity to derive high-affinity scFvs against several target antigens.

T 403 AN IN VIVO MODEL FOR DETERMINING RULES OF ANTIBODY DESIGN, J Isaacs, J Greenwod and H Waldmann, Cambridge University Dept of Pathology, Tennis Court Road, Cambridge, U.K.

In vitro data cannot help us to decide which of the various possible effector mechanisms are utilised by monoclonal antibodies *in vivo*, and without this information rational antibody design is not feasible. We have previously described a mouse model for assessing the lytic capacity of engineered antibodies *in vivo*, based upon the clearance of CD8+ lymphocytes from peripheral blood. We have now tested mutant antibodies in this model to determine the importance of complement and Fc receptor mediated clearance. Our results suggest a dominant role for Fc receptors. Thus, whereas interference with complement activation does not usually impair target cell clearance, prevention of Fc receptor binding can render an antibody impotent in this regard. The data also suggest redundancy amongst the different groups of Fc receptors.

In addition to suggesting designs for purely 'blocking' antibodies our model has also proved useful in the identification of 'super-antibodies' with improved lytic capacity *in vivo*, and these data will be presented at the meeting.

T 404 CONSTRUCTION AND PROPERTIES OF BISPECIFIC DIABODY MOLECULES WITH VARYING LINKER LENGTHS BETWEEN VARIABLE DOMAINS Ronald H. Jackson, Anthony R. Pope, Philipp Holliger*, Terence Prospero* and Greg Winter* Cambridge Antibody Technology, The Science Park, Melbourn, Cambridgeshire, SG8 6EJ and * MRC Centre for Protein Engineering, Hills Road, Cambridge, CB2 2QH, U.K.

Diabodies are small antibody fragments with two antigen binding sites (P. Holliger, T. Prospero and G. Winter Proc. Natl. Acad. Sci. USA 90 6444-6448 1993). The fragments comprise a heavy-chain variable domain (VH) connected to a light chain variable domain (VL) on the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen binding sites. For a bispecific diabody directed against lysozyme and oxazolone, it was demonstrated that those with 5 and 15 amino acid linkers had similar binding affinities to the parent antibodies but a fragment with the VH domain joined directly to the VL domain was found to have an improved affinity for antigen. We will report the extension of these studies to determine the effect of varying the linker length on the expression and affinity of bispecific diabodies including examples where deletions have been made to the termini at the junction between domains in the VH-VL polypeptide. Such constructs further reduce the flexibility of the constrained diabody molecule. Several constructs of a diabody directed against lysozyme and oxazolone have been made, each with a different 1 amino acid deletion at the junction between VH and VL domains and also further constructs with 2 and 3 amino acid deletions. The properties of these molecules will be discussed.

T 405 DESIGN OF INTERCHAIN DISULFIDE BONDS IN THE FRAMEWORK OF FV FRAGMENTS S-H. Jung, I. Pastan and BK Lee, Lab. of Mol. Biol., Natl. Cancer Institute, NIH, Bethesda, MD 20892

Fv fragments are the smallest units of antibodies that retain the specific antigen binding characteristics of the whole antibody and are being used for the diagnosis and therapy of human diseases. These Fv's are non-covalently associated heterodimers of the VH and VL domains and are unstable. A Fv is usually stabilized by connecting the two domains by a peptide linker (Bird et al, Science *242*, 423, 1988; Huston et al, PNAS *85*, 5879, 1988). An alternative strategy is to connect the two chains by means of an interchain disulfide bond. We used molecular modeling tools to identify possible interchain disulfide bond sites in the framework (FR) region of the Fv model structure of the monoclonal mouse antibody (mAb) B3 (Pastan et al, Cancer Res. *51*, 3781, 1991). Two sites were identified; VH44-VL105 and VH111-VL48. (VH44-VL100 and VH105-VL43 in the numbering scheme of Kabat et al., "Sequence of Proteins of Immunological Interest", 1991.) These sites in Fv are related by a 2-fold pseudo-rotational symmetry. The selection criteria and the modeling scheme will be described. Because these disulfide bond sites are in the framework region, they can be located for the Fv of any immunoglobulin molecule from sequence alignment alone. To test this design, interchain disulfide bonds were introduced by site-directed mutagenesis in the Fv fragments of three different antibodies, mAb B3 (Brinkmann et al., PNAS, *90*, 7538, 1993), e23, and anti-Tac, each fused to a truncated form of *Pseudomonas* exotoxin. All three chimeric toxins were found to be just as active as the corresponding single chain counterparts and considerably more stable. The purification yield was also significantly higher (see Poster by Brinkmann et al).

T 406 **MOLECULAR MODELLING OF THE LIKELY INTERACTIONS BETWEEN CD4 AND IMMUNOGLOBULIN VARIABLE DOMAINS IMPLICATES THE V_H FRAMEWORK SURFACE AS THE LIKELY SITE OF BINDING .** Perry M. Kirkham[1], Maurizio Zanetti[2], and Harry W. Schroeder, Jr.[1], [1]Departments of Microbiology and Medicine, University of Alabama at Birmingham, Birmingham, AL 35294-3300, and [2]Department of Medicine and Cancer Center, University of California, San Diego, La Jolla, CA 92093-0961.

The T cell surface glycoprotein CD4 serves as a co-receptor for T cell activation and as a ligand for the HIV glycoprotein gp120. One of us has previously shown that CD4 also binds polyclonal human immunoglobulin in a manner independent of heavy or light chain isotype. Binding patterns implicate a highly family-specific and evolutionarily conserved VH framework (FR) 1 and 3 subdomain on the antibody surface as a likely binding site for CD4. We have examined this possibility using molecular modeling to analyze the ability of this FR1/FR3 region to serve as a receptor-ligand participant. In addition, we have used the software package DOCK 3.0 to identify potential docking sites for CD4 on the antibody. The results of these analyses demonstrate that the total area of potential contact, number of residues involved, and total charge-charge interactions are compatible with those for known protein-ligand complexes. Because this antibody VH FR region is family-specific in character, we predict that CD4 will bind selectively to certain VH families and mammalian clans of evolutionary relatedness. Because of the importance of CD4 as a co-receptor for T cell activation, and due to its vital role in the pathogenesis of AIDS, localization of a binding site between CD4 and the antibody molecule is an important first step to an understanding of how CD4 contributes to the development of the humoral response.

T 407 STRUCTURAL ANALYSIS OF A PHOSPHORYLCHOLINE-BINDING ANTIBODY WITH A UNIQUE CARRIER SPECIFICITY FOR TRICHINELLA SPIRALIS, Pak-Leong Lim, D.T.M.Leung, Y.L.Chui and C.H.Ma, Clinical Immunology Unit, Chinese University of Hong Kong, Hong Kong

A phosphorylcholine(PC)-binding IgG antibody (Mab2) produced by a hybridoma derived from a BALB\c mouse which had been immunized against Trichinella spiralis was found to bind only to the immunizing antigen (TSC) but not to other PC-associated antigens such as PC-OVA. Sequence analysis revealed the presence of a heavy chain (VH) which was very similar (except for four aminoacids) to that of the M511 myeloma protein, and a light chain which was completely identical to that of the M167 myeloma protein. Unlike Mab2, both M511 and M167 as well as TEPC 15 and W3207 (two other PC-binding proteins) were indiscriminate in their binding. In addition, three M511/M167-like hybridoma antibodies were found to bind best to PC-OVA and least to TSC. A comparison of the VH sequences of all these proteins revealed the glycine substitution at 49H in Mab2 to be the single most important change which could contribute to the unique specificity of the molecule. All other PC-binding proteins reported to-date have alanine instead of glycine at this position. Site-directed mutagenesis will be used to verify the importance of this substitution in Mab2.

T 408 DEFECTIVE SECRETION OF AN IMMUNOGLOBULIN CAUSED BY MUTATIONS IN THE HEAVY CHAIN CDR2, T. M. Martin, Q. Chen, S. Stevens, M.B. Rittenberg, Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, OR 97201

The assembly and transport of immunoglobulins is a useful model to analyze the mechanisms which control protein synthesis and secretion. Through analyses of mutant antibodies with defects in secretion, various regions of immunoglobulin molecules are known to play a role in proper folding, assembly and transport of these proteins. So far, these regions have been confined to the constant domains or to invariant or highly conserved amino acids of the variable domains. Here we show for the first time that random point mutations in an immunoglobulin hypervariable region can affect secretion. These mutants were generated via random mutagenesis of the VH CDR2 region of the anti-phosphocholine antibody, T15. The low secretor mutants differ from WT T15 by only 2-4 amino acids in VH CDR2, but they secrete less than 10% of the wild type level of immunoglobulin. Production of mRNA and intracellular protein is normal while assembly appears inefficient upon ELISA analysis of cellular lysates. One mutant has a significant, but low, amount of assembled intracellular Ig which can bind PC-protein, indicating a transport defect as well. The other low secretors may be much more deficient in assembly. The low secretor phenotype from VH CDR2 mutants is surprising because the process of affinity maturation during an immune response requires somatic mutation of CDRs and therefore mutations in these regions would be expected to be highly permissive. (Supported by NIH Grants AI14985 and 26827 and a Tartar Foundation Research award to TMM)

T 409 INFLUENCE OF HINGE-STRUCTURE ON EFFECTOR FUNCTIONS: ONE S-S BOND IN FRONT OF THE C_H2 DOMAIN IS NECESSARY AND SUFFICIENT FOR EFFECTOR FUNCTION OF HUMAN IGG3 WITHOUT A GENETIC HINGE, Terje E Michaelsen1, Audun Aase[1], Randi H Sandin[1], Ole H Brekke[2], Bjørn Bremnes[2], Lars Norderhaug[2] and Inger Sandlie[2]. Department of Vaccines[1], Natl. Inst. Public Health, Oslo and Molecular Biology[2], Institute of Biology, University of Oslo, Norway.

We have previously demonstrated that the IgG3 molecule do not need the whole 62 amino acids hinge region in order to mediate effector functions, since several mutants with 12-15 amino acids hinge regions can be up to 100 times more efficient in inducing complement mediated cell lysis (CML) than the IgG3 wild type. In order to determine the minimum hinge structure for effector functions, we have created four IgG3 mutants with the same NIP-binding activities: 1) an IgG3 molecule without a hinge (OsNP05), 2) an IgG3 molecule without a hinge,but with the Cys 131 residue in the heavy chain mutated to Ser thereby permitting the L-chains to S-S bond to each other (OsNP14), 3) an IgG3 molecule without a hinge, but with the insertion by mutagenesis of a Cys residue in front of the C_H2 between Ala 231 and Pro 232 (OsNP10), and 4) an hybrid between OsNP10 and OsNP14 were the IgG3 molecule is without a hinge, has Ser in position 131 and Cys inserted between Ala 231 and Pro 232 (OsNP40). All these mutants were tested in CML , ADCC (antibody dependent cell mediated cytotoxicity) by peripheral blood leucocytes and phagocytosis or respiratory burst mediated by U-937 or peripheral blood neutrophils and compared with IgG3 wild type and mutant OsNP03 (with 15 aa hinge). The mutants OsNP10 and OsNP40 was active in CML, ADCC, phagocytosis and respiratory burst, while the OsNP05 and OsNP14 mutants were negative in CML, ADCC and in phagocytosis mediated by FcRI on U-937 and negative or slightly positive in respiratory burst mediated by peripheral blood neutrophils from normal donors. - The results indicate that a hinge region is not necessary as a spacer between Fab and Fc, but a correct alignment of the two C_H2 regions by minimum 1 S-S bond is necessary and sufficient for effector functions. If the two C_H2 regions are align to each other by an L-L chain S-S bond as in OsNP14 or drifted apart as in OsNP05, the molecule get inactive in effector functions due to improper C_H2 quarterary structure/conformation.

T 410 ANTIBODY STRUCTURE ANALYSIS WITH THE ANTIBODY STRUCTURAL DATABASE (ASD), Victoria A. Roberts, Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037.

Understanding antibody structure provides a basis for rational design of antibodies. Unfortunately, the structures for only a very small fraction of antibodies in the antibody repertoire will ever be determined by X-ray crystallography or NMR techniques. However, the many antibody structures that have been determined to date provide a structural basis for analyzing and predicting antibody structure. We have constructed a visual database of antibody structures (the ASD) that contains separately superimposed variable light and variable heavy chains, which reveal not only conserved backbone structure, but also structurally conserved side-chain conformations. The intact variable region of each antibody is also included for the investigation of the geometry of the light chain/heavy chain interface. Several applications of the ASD will be discussed. Structural analysis has revealed that regions of the loops forming the antigen-binding site are structurally conserved, despite sequential variation. Six sites in these structurally conserved regions have geometries similar to the zinc-binding site of the enzyme carbonic anhydrase. Appropriate substitutions in four of these sites in three different antibodies have resulted in copper and zinc binding antibodies. Thus, this method of template-based design is general and, in addition, may help evaluate the function of residues in binding motifs independently of the other features of the evolved enzyme active site. The ASD has also been used to construct models of the variable region of antibodies. Models of phosphorylcholine-binding antibodies have suggested that that related antibodies may bind antigen in different modes and have been used to interpret results from random mutagenesis experiments. The model of a catalytic antibody has predicted roles for specific side chains in catalysis and antigen binding, which have been tested by site-directed mutagenesis. The ASD also provides rapid evaluation of antibody models built by other techniques by allowing rapid comparison with the known structures. Thus, the ASD provides a general tool for evaluation, analysis, and prediction of antibody structure.

T 411 Biologic Properties of an Anti-Carcinoma CH2 Domain-Deleted Recombinant Immunoglobulin

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Monoclonal antibody CC49 is a second generation mAb that reacts with TAG-72, a high molecular weight mucin expressed on the surface of a variety of carcinomas. Currently, CC49 is being evaluated in clinical trials for its use in the diagnosis and treatment of cancer. Because of problems associated with the human anti-mouse antibody (HAMA) response and slow clearance from the blood pool which may lead to toxicity of normal tissue, we designed a chimeric recombinant CH2 domain-deleted antibody to eliminate or reduce these problems. The murine variable region of CC49 was cassetted into an expression vector containing the human constant regions with a CH2 deletion. The vector was transfected into a murine myeloma previously transfected with the chimeric light chain of CC49. The resulting cCC49ΔCH2 mAb was then compared to intact cCC49. Competition radioimmunoassays demonstrated that cCC49ΔCH2 had a relative affinity similar to that of cCC49. Pharmacokinetic studies in athymic mice showed that ^{131}I-cCC49ΔCH2 had a significantly faster clearance out of the blood pool than did ^{125}I-cCC49. Biodistribution studies, using human colon carcinoma xenografts in athymic mice and iodine-labeled cCC49ΔCH2 or cCC49 demonstrated that both cMAbs were able to localize to tumors. The iodine-labeled cCC49ΔCH2 had a reduced percentage of the injected dose in all tissues, including kidney, in comparison with cCC49. Although the percentage of the injected dose in the tumor was less overall for cCC49ΔCH2, the tumor to tissue ratios were much higher because of the rapid plasma clearance and reduced normal tissue binding. Thus, because of its rapid plasma clearance, lower normal tissue uptake, faster and relatively high tumor localization, iodinated cCC49ΔCH2 shows potential for diagnostic and perhaps therapeutic applications

T 412 DESIGN, PRODUCTION AND CHARACTERIZATION OF RECOMBINANT POLYMERIC IgG, Richard I.F. Smith and Sherie L. Morrison, Dept. of Microbiology and Molecular Genetics, University of California, Los Angeles, CA 90024

IgM and IgG are the only classes of immunoglobulin capable of initiating the classical complement cascade. IgM, however, fixes complement much more effectively than IgG making it a more attractive Fc for some immunotherapeutic strategies. On the other hand, IgG has a longer serum half-life than IgM and triggers additional immune responses mediated through γ specific Fc receptors. By endowing human IgG with structural features shown to be involved in IgM polymer formation we hoped to produce IgM-like polymers of IgG that would possess the desirable properties of IgG in the more potent, polymeric form of IgM. Here we describe the successful engineering and purification of polymeric IgG secreted from transfectants of a murine myeloma derived cell line. These polymers have proven to be as effective as IgM in complement mediated lysis and more efficient at binding C1q, the first component of complement, than both IgG and IgM. This latter observation clearly illustrates the point that C1q binding alone does not determine the ability of a molecule to fix complement. In addition, studies using ^{125}I-labeled protein indicate that polymerization does not interfere with recognition by FcγRI on the surface of U937 cells nor does it affect serum half-life.

T 413 ENGINEERING AND ANALYSIS OF PRESSURE SENSITIVE ANTIBODIES, Srikanth Sundaram*, Suzanne Scarlata❖, David M. Yarmush*, and Martin L. Yarmush*, *Department of Chemical and Biochemical Engineering, Rutgers University, Piscataway, NJ 08855-0909, ❖ Department of Physiology and Biophysics, State University of New York, Stony Brook, NY 11794

Antibodies are subjects of major interest for molecular modeling and protein engineering studies due to their wide diversity, homogeneity and potential for modification for clinical and industrial use. In all these applications, understanding antibody-antigen recognition and binding mechanisms may play a crucial role in the development of operational strategies. We are using antibody engineering as a tool to study, at the molecular level, the mechanism of pressure-induced dissociation of antigen-antibody complexes.

A significant problem in the large scale use of immunoadsorption has been the lack of a mild elution scheme that completely preserves the activity of both antigen and the immunoadsorbent over many uses. We have shown that pressure can be a mild and effective method for recovering antigens from immunoadsorbents. For one of the immunoadsorbents used in this study, over 75% of the reversibly bound antigen was recovered following a single incubation at 2000 atm and over 90% recoveries were obtained by repeated pressurizations. Repeated pressurizations to 2000 atm exerted no detrimental effect on immunoadsorbent binding properties, whereas immunoadsorbent binding capacity was significantly reduced upon treatment with a common eluent (glycine/HCl at pH 2.5).

In our current studies, pressure effects are being evaluated by computing volumetric changes (ΔV) accompanying antigen-antibody reactions using high pressure fluorescence polarization spectroscopy. Intact antibodies as well as proteolytically or bacterially produced antigen-binding fragments of antibody molecules such as the Fab fragment and the single-chain antibody fragment (sFv) are being studied. Thermodynamic parameters including enthalpies and entropies will be generated by carrying out pressure experiments at varying temperatures which will provide additional insight with regards to the mechanism of pressure-induced dissociation of protein-protein systems. These studies provide the basis for future protein engineering investigations into the role of the various intermolecular interactions and the variable-domain interface in the pressure-induced behavior of antigen-antibody systems.

Expression of MAbs and MAb Derivatives in Prokaryotic, Eukaryotic and Transgenic Systems; Clinical and Diagnostic Experience with Genetically Engineered MAbs

T 500 A DIABODY FOR IMAGING OF ATHEROSCLEROTIC PLAQUES, J. Adair[1], K., Armour[1], F. Carr[1], C. Ditlow[2], F. Chen[2], P. Holliger[3], G. Winter[3] and W. Harris[1],[1]Scotgen Biopharmaceuticals Inc., Research Centre, Kettock Lodge, Aberdeen Science and Technology Park, Bridge of Don, Aberdeen, AB22 8GU, Scotland, [2]Scotgen Biopharmaceuticals Inc., Corporate Headquarters, 1014 Menlo Park, CA 94025; [3]Medical Research Council, Centre for Protein Engineering, Hills Road, Cambridge, CB2 2QH, England.

Cardiovascular disease is the major cause of morbidity and mortality in the USA and western countries. Each year approx. 1.5 million Americans have heart attacks and 500 000 die yearly of which approx. 300 000 never reach hospital. Atherosclerosis, characterized by the thickening and "hardening" of the arterial wall, is a significant contributor to cardiovascular dysfunction. MAb Z2D3 (IgM/k) recognizes a novel antigen presented on atherosclerotic plaque and has potential for imaging and therapy of atherosclerosis. For imaging a molecule of low molecular weight would be preferred because of the advantage of rapid clearance from the rest of the body and penetration to antigen throughout the plaque. Monomeric antibody fragments such as Fab, Fv or single-chain Fv (scFv) usually lead to lower binding avidity, often of up to an order of magnitude, compared to bivalent F(ab')$_2$ or IgG. Therefore a bivalent, low molecular weight, alternative which could be constructed without the need for chemical cross-linking would be would be a useful entity.

It has been recently demonstrated that when scFv with short (<10 amino acids) peptide linkers are expressed in bacteria a significant proportion of the scFv spontaneously dimerizes and that the dimeric product, termed "diabody", has antigen binding activity similar to that seen for whole IgG. Diabodies are the predominant product when the peptide linker is removed and the two domains of the scFv are directly linked. We have constructed a diabody using the variable regions from antibody Z2D3. The diabody has a binding affinity for antigen in plaque similar to that of chimeric mouse variable region human IgG1 constant region Z2D3 antibody and a chimeric F(ab')$_2$ fragment. The Z2D3 diabody has been used to recognize antigen in histological specimens. This novel molecule could be used as a radio-imaging or MRI agent for localization in vivo of coronary, cerebral and peripheral lesions and for estimation of the probability of restenosis.

T 501 THE ENGINEERING OF (PL)ANTIBODIES FOR VIRUS PROTECTION IN TRANSGENIC PLANTS Eugenio Benvenuto, ENEA, Settore Biotecnologie ed Agricoltura, C.P. 2400, 00100 Roma (ITALY).

Since the first demonstration that plants are able to produce antibodies, plant pathologists have been attracted by the possibility to exploit the immune-system repertoire to confer new forms of disease resistance to plants. In fact, the "immunomodulation" of selected antigens involved in pathogenesis could lead to transgenic plants "genetically immunized". Although previous works have shown the possibility of expressing the complete antibody molecules in plants, engineered forms of immunoglobulin such as single chain antibodies appear to be more appropriate for expression in plants particularly for their small size and the lack of assembly requirements.

Here the first demonstration of the functional arrest of a plant viral infection mediated by a scFv antibody constitutively expressed in plants is reported. In this work the coat protein of the Tombusvirus AMCV (Artichoke Mottled Crinkle Virus) was chosen as an ideal molecule to interfere with. The engineered scFv antibody derived from a monoclonal antibody that recognized a higly conserved epitope of the coat protein is stably expressed intracellularly. Transgenic *Nicotiana benthamiana* plants challenged with the virus show a drastic reduction of infection incidence and delay in symptom development.

The feasibility of designing of antibodies that bind and inactivate key molecules involved in the development of some plant diseases is discussed.

T 502 CONSTRUCTION AND BACTERIAL EXPRESSION OF AN ACTIVE SINGLE CHAIN FV ANTIBODY SPECIFIC FOR SCORPION HEMOCYANINS, P. BILLIALD and D. J. VAUX, Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE (UK)

A58 is a murine monoclonal antibody raised against the hemocyanin of the Tunisian scorpion *Androctonus australis*. A58 binds an internal dimeric subunit of the hemocyanin with such high affinity that the native hemocyanin disaggregates upon antibody binding, as shown by immunoelectron microscopy. Moreover, A58 is highly selective and exhibits cross-reactivity with a limited number of scorpions species. All of them belong to a single Family (Buthidae), which is the main Family with regard to its number of species (40%) as well as its medical interest. We have constructed synthetic genes encoding single-chain antigen binding protein (scFv) related to A58. Different bacterial expression systems and conditions of inductions were investigated for expression of a periplasmic recombinant protein. Growth at temperature higher than 30 degrees C resulted in an accumulation of degradation products while lower temperatures favoured the accumulation of undegraded soluble and active protein exported to the periplasm and secreted in the culture medium. The scFv has similar binding properties to the original murine antibody. It binds specifically the dimeric subunit of *Androctonus australis* hemocyanin, cross-reacts with only one subunit of each Buthidae investigated and blocks the recognition of the hemocyanin by A58 antibody in competitive ELISA. Its affinity to hemocyanin, compared to the parent antibody, the single A58 VH domain and a scFv containing point mutation in the canonical structure of the VL domain, is now being investigated.

Previous reports have demonstrated the value of monoclonal antibodies in taxonomical and molecular evolution studies. scFvA58 is the first of a new generation of antigen binding proteins which should prove useful in Scorpions taxonomical studies as well as for the investigation of the VH-VL structure-function relationship of antibodies.

T 503 **DISULFIDE STABILIZED FV FRAGMENTS (dsFV) AND dsFV-IMMUNOTOXINS** U.Brinkmann,Y.Reiter, S-H.Jung, K.Webber, BK. Lee and I.Pastan, Laboratory of Molecular Biology, Natl. Cancer Institutes, Natl. Institutes of Health.

Fv fragments are unstable VH-VL heterodimers.This instability can be overcome by connecting V_H and VL by a peptide linker (1,2). Such single-chain Fvs (scFvs) retain specificity and affinity and are used for imaging tumors. When producing immunotoxins composed of Fv's and truncated forms of Pseudomonas exotoxin (that are specifically cytotoxicity towards target cells in vitro and can cure human carcinomas in mice, 3-5), we encountered several "problems" with scFv's and scFv-immunotoxins: (i) they sometimes have reduced affinity, (ii) they are not easily produced in large amounts and (iii) they often are unstable and aggregate. We developed an alternative approach to stabilize the Fv heterodimer by introducing interchain disulfides between VH and VL (6). The appropriate (cys)-positions in conserved framework regions of VH and VL that are distant from the CDR's and should be able to disulfide-stabilize any given Fv without affecting its structure or antigen binding were identified by computer modeling. We made various disulfide-stabilized (two-chain) Fv's (dsFv's) and dsFv-immunotoxins, directed against a carcinoma related carbohydrate, B3(dsFv); the IL2-R[p55], antiTac(dsFv); and erbB2, e23(dsFv). The affinity of the dsFv's and/or the specific cytotoxicity in vitro and anti-tumor activity in vivo of dsFv-immunotoxins was the same or better than that of the correspondent scFv and -immunotoxins. We found, that in all of the "weak spots" of scFv's (stability, production, affinity) the dsFv's are superior:

(i) dsFv's and -fusion proteins are much more stable against thermal or chemical denaturation, (ii) dsFv's and dsFv-immunotoxins are produced with much higher yields, and (iii) the reduced affinity of scFv's compared to IgG or Fab'(e.g. in e23(scFv)-immunotoxins) can be improved using the dsFv.

1) Bird et al. Science 242, 423, 1988; 2) Huston et al. PNAS 85, 5879, 1988; 3) Brinkmann et al. PNAS 88, 8616, 1991; 4) Batra et al. PNAS 89, 5867, 1992; 5) Chaudhary et al. Nature 339, 394, 1989; 6) Brinkmann et al. PNAS 90, 7538, 1993.

T 504 **MANIPULATION OF T-CELL SPECIFICITY FOR THERAPEUTICAL ENDS,** Thomas Brocker, Andre Traunecker, Klaus Karjalainen, Basel Institute for Immunology, CH-4005 Basel, Switzerland

The recognition of antigen and the transduction of activating signals into the T cell are mediated by several different components of the TCR/CD3-multiprotein complex. We wanted to bypass this complexity by creating a chimaeric molecule that would retain both signalling- and recognitionfunctions within one polypeptide chain in order to generate an easily manipulatable system, which would allow us to change the T cell specificity at will.

Based on experiments which indicated that a major signalling role in the TCR/CD3-complex is played by the CD3ζ-chain, we constructed a molecule that is composed of the cytoplasmic and transmembrane portion of the CD3ζ-chain, a short hinge region and a single chain combining site (Fv) derived from monoclonal antibodies.

When transfected into Tcr-negative or Tcr-positive T cell hybridomas, this chimaeric molecule is able to mediate recognition of antigen expressing cells and the observed signaltransduction results in production of IL-2 and IL-3 by the transfectants.

Since the antigen specificity of our model-chimaera can be changed easily by replacing its Fv-domain with other Fvs derived for example from tumor-antigen specific antibodies, this system could be useful to create T cells of any desired (tumor-) specificity bypassing the necessity of Mhc-restriction.

The creation of transgenic mice as a constant source of T-lymphocytes and NK-cells expressing this chimeric Tcr allows us to test the properties of this protein in an animal-tumor model.

T 505 CHARACTERIZATION OF THE SINGLE Cα GENE OF SWINE, William R. Brown and John E. Butler, Department of Microbiology, University of Iowa, Iowa City, IA 52242

The cDNA sequence encoding the constant region of the porcine IgA heavy chain as well as its genomic organization, has been determined. A cDNA clone (1A1) spanning the CH3 domain and part of the CH2 domain was isolated from a porcine mesenteric lymph node cDNA library. Clone 1A1 was aligned with a PCR-generated DNA fragment encompassing the CH1 domain through the 5' end of the CH3 domain to derive the complete cDNA sequence. Comparison with other mammalian Cα heavy chains (hinge regions excluded) indicated that the deduced amino acid sequence of porcine Cα is most homologous with the human Cα subclasses (>70%), followed by mouse Cα (61%) and a consensus sequence of the thirteen rabbit Cα heavy chains (59%). The greatest sequence homology was found among the CH3 domains in all species. A striking feature of porcine Cα is its short six amino acid hinge which like other mammalian IgAs, is encoded within the CH2 domain. Sequence analysis of germline Cα, generated by PCR from liver or sperm DNA, revealed an exon-intron organization similar to other mammalian Cα genes. Genomic Southern blot analysis indicates that only a single copy of Cα exists within the porcine genome. Data obtained in these studies will be valuable in pursuing the use of swine as a model in immunological research. Furthermore, due to the high degree of homology between porcine and human IgA at the protein level, the possibility exists for using porcine IgA as a passive antibody in humans.

T 506 EXPRESSION ANALYSIS OF A MAK33 SINGLE-CHAIN Fv PROTEIN IN *NICOTIANA TABACUM*, Anne-Marie Bruyns, Myriam De Neve, Isabelle Strobbe, Anni Jacobs, Marc Van Montagu and Ann Depicker, Laboratorium voor Genetica, Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

To evaluate the efficiency of inhibition of an enzyme in the plant cell by expression of an inhibiting antibody, we chose as a model system the human enzyme creatine kinase-MM (CK-MM) and the mouse monoclonal antibody MAK33 that inhibits this enzyme by 80%. We decided to work with two systems in parallel namely one with translocation of both components to the endoplasmic reticulum and one with expression of both components in the cytoplasm.

The cDNA for the creatine kinase subunit M has been cloned in the expression cassette of a T-DNA vector, with and without the 2S2 signal peptide. These T-DNAs have been introduced into *Nicotiana tabacum* by *Agrobacterium tumefaciens*-mediated transformation and GV-CK and GV-SSCK transgenic plants were selected on phosphinothricin. The creatine kinase expression level in these transgenic plants will be evaluated at the RNA and protein level.

For the expression of the MAK33 component, a MAK33 single-chain Fv-encoding sequence (*scFv*) was constructed by successive PCR reactions on the basis of the cDNAs for the light and heavy chain. In order to compare the intra- and extracellular accumulation of this scFv, a derived construction was made in which the *scFv* was fused with the 2S2 signal peptide. Both *scFv*-encoding sequences were cloned in a plant expression cassette; the chimeric genes were subsequently introduced into a T-DNA vector with a hygromycin resistance marker. These T-DNAs have been introduced into *N. tabacum* by *A. tumefaciens*-mediated transformation and the GV-AS and GV-SSAS transgenic plants were obtained. The *scFv* expression level in these transgenic plants will be evaluated at the RNA and protein level.

Subsequently, we will cross the GV-AS expressing plants with the GV-CK expressing plants and the GV-SSAS expressing plants with the GV-SSCK expressing plants to evaluate the interaction of the MAK33 scFv with its antigen CK-MM.

T 507 A HUMANIZED, BISPECIFIC IMMUNOADHESIN-ANTIBODY THAT RETARGETS CD3+ EFFECTOR CELLS TO KILL CELLS INFECTED WITH HIV-1, Steven M. Chamow, Dezhen Zhang*, Shilpa M. Mhatre*, Xiang Y. Tan*, Scot A. Marsters, David H. Peers, Randal A. Byrn*, Avi Ashkenazi†, and Richard P. Junghans*, Departments of Process Science and Molecular Biology†, Genentech, Inc., 460 Pt. San Bruno Blvd., S. San Francisco, CA 94080; Division of Hematology/Oncology*, Harvard Medical School, New England Deaconess Hospital, Boston, MA 02215

We have developed a humanized, bispecific immunoadhesin-antibody (BIA) that targets and kills HIV-infected cells. Comprised of CD4-IgG and humanized anti-CD3 IgG, this BIA is bifunctional: first, it exploits the natural affinity of CD4 for gp120 to target the BIA to HIV-infected cells; and second, it recruits and activates, through its anti-CD3 moiety, cytotoxic T lymphocytes (CTL) to lyse target cells in a non-MHC restricted manner. To produce purified BIA from supernantants of transfected mammalian cells, we designed a three-step recovery scheme based on the structural elements of this heterotrimeric protein. The ability of purified BIA to specifically lyse HIV-infected target cells was demonstrated using CTL from two different sources: whole peripheral blood lymphocyte (PBL) fractions, or pure CTL preparations. In contrast, a human anti-gp120 antibody mediated lysis of HIV-infected traget cells only with PBL fractions and not with purifed CTL. Moreover, lysis observed in the presence of the human anti-gp120 antibody was completely blocked in the presence of human serum (which competes for Fcγ receptor binding), while BIA-mediated lysis of target cells was not affected. We measured the monovalent affinities of BIA for HIV-gp120 on infected cells and for CD3ε on CTL. Relative to the bivalent parent molecules, CD4/gp120 affinity in the BIA is unchanged, while anti-CD3/CD3 is substantially decreased. We further demonstrated by fluorescence microscopy that physical association of CD3+ cells with gp120-expressing cells occurs only in the presence of BIA. Thus, the cytocidal activity of BIA in the presence of serum reflects its unique ability to recruit CTL as effector cells, and highlights a potentially important advantage of this type of construct over antibodies for HIV-directed therapy.

T 508 BIVALENT AND BISPECIFIC DIABODIES FOR COLORECTAL TUMOUR TARGETING.

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Diabodies are small bivalent or bispecific antibody fragments suitable for expression directly from bacteria (Holliger *et al.*, *Proc. Natl. Acad. Sci.* USA 90:6444-6448, 1993)

Here we describe the construction and characterisation of bivalent anti-carcinoembryonic antigen (CEA) and anti-FcγRIII (CD16) diabodies, and the generation of a bispecific anti-CEA x anti-CD16 diabody. The antibody V genes were derived from the hybridoma 3G8 (anti-CD16) and from a phage display library (anti-CEA) and were formatted initially as scFv fragments. They were converted to diabody format by shortening the sc-linker from fifteen to five amino-acids (GGGGS) which prevents the intrachain pairing of VH and VL domains, thus favouring the formation of dimers. The diabodies bind to their target antigens as demonstrated by ELISA and FACS analysis. BIACORE measurements confirm that the bivalent anti-CEA diabody (biv-αCEA) has an affinity of about 3 nM which is the same as the initial scFv (sc-αCEA). The affinity of the bivalent anti-CD16 diabody (biv-αCD16) is approximately 10 nM.

The VL domains of biv-αCEA and biv-αCD16 were then exchanged to create two complementary polypeptides, each comprising two antibody V domains : VHαCD16-VLαCEA and VHαCEA-VLαCD16. Neither polypeptide binds to antigen alone, but together they form an active heterodimer. The two (myc-tagged) polypeptides were expressed from a single bicistronic pUC119-based vector transfected into *Escherichi coli* and functional diabody was extracted from periplasm or from culture supernatant. Detailed characterisation of the purified bispecifc diabody (bis-αCD16-αCEA) confirms that it binds to both CEA and CD16 (as purified antigen or on living cells) with similar kinetics to the parent diabodies. ELISA demonstrates that bis-αCD16-αCEA can bind simultaneously to CEA and to recombinant human CD16.

The diabodies biv-αCEA and bis-αCD16-αCEA have been purified on a CEA column. Experiments are underway to demonstrate bis-αCD16-αCEA diabody directed lysis of colorectal cell lines by LAK cells and to examine the *in vivo* localisation of biv-αCEA diabody to colorectal tumour xenografts. Bispecific diabodies promise to be powerful reagents to target cell destruction utilising natural cellular effector mechanisms.

T 509 CLONING AND EXPRESSION OF A HUMAN TYPE V ACID PHOSPHATASE SINGLE CHAIN ANTIBODY FRAGMENT IN *E. coli*, Pauline M. Cupit, Andrew J. R. Porter, Michael J. Brown*, Janet E Carey*, Steve Holmes* and William J. Harris, Department of Molecular and Cell Biology, Marischal College, University of Aberdeen, Aberdeen, Scotland, UK and *SmithKline Beecham, Pharmaceuticals Research and Development, Department of Biotechnology, Harlow, England, UK.

Human type V acid phosphatase belongs to a distinct class of purple iron containing proteins and is characterised by its resistance to inhibition by tartrate. It is, therefore, often referred to as TRAP (Tartrate Resistant Acid Phosphatase). TRAP is present in a wide variety of tissues and has been used as marker to identify osteoclasts and their precursors in bone. High level expression of TRAP in the bone resorptive area suggests a functional involvement of the enzyme in the bone resorption process. Our aim is to develop a single chain antibody fragment directed against TRAP as the basis for a highly sensitive and specific immunoassay for skeletal TRAP as a diagnostic indicator for osteoporosis.

cDNA encoding the heavy (VH) and light (VL) chain variable domain genes of an anti-TRAP monoclonal antibody were cloned and sequenced using standard techniques. The anti-TRAP single chain antibody expression vector was constructed by inserting the VH and VL domain genes into the plasmid pPM1. The single chain fragment expressed from pPM1 consists of the VH domain linked to the VL - CL domains by a 14 amino acid linker. In addition, a 6 x His tag is fused to the CL domain to facilitate a one step purification of the fragment. The anti-TRAP vector construct was expressed in *E.coli* and results will be presented comparing the affinity of the single chain fragment for TRAP with that of the parent monoclonal antibody.

T 510 ENZYME AND SPECIFIC ANTIBODY FRAGMENT INTERACTIONS IN THE CYTOPLASM OF PLANT CELLS, Geert De Jaeger, Anton Gerats, Marc Van Montagu and Ann Depicker, Laboratorium voor Genetica, Universiteit Gent, B-9000 Gent, Belgium

Expression of specific antibody fragments (ScFv) in plant cells could become a powerful approach in plant improvement. Inhibition of a key protein in a plant/pathogen interaction could result in pathogen-resistant plants. Moreover, the production of undesired metabolites could be lowered by inhibiting a specific enzyme in the metabolic pathway. As a model system, the flavonoid biosynthetic pathway was chosen that is responsible for the color of the flower. We are testing whether an enzyme/ScFv interaction can switch off this pathway in *Petunia hybrida*. This pathway has proven to be an excellent model system to study inhibition of gene expression by using cosuppression or anti-sense RNA. The enzyme we are focusing on, is dihydroflavonol-4-reductase (DFR). DFR is one of the key structural enzymes in the flavonoid pathway of *P. hybrida*. Besides the DFR protein itself, which will be overproduced in bacteria, synthetic oligopeptides were made to raise an immune response. These peptides were coupled to the carrier protein keyhole limpet hemocyanin (KLH) and injected into rabbits. Native immunoblot analysis showed that only one of the three oligopeptides encodes a sequential epitope of the native DFR. Three different mouse lines were subsequently injected with this KLH-coupled peptide. Currently, a scantibody-f_d phage library is being constructed on the basis of the B-lymphocytes isolated from mouse lymph nodes. By panning against the peptide, the library will be enriched for peptide-binding f_d clones and these clones will be screened for DFR binding. Subsequently the DFR-binding ScFv fragments will be assessed for their inhibition of the enzyme *in vitro* by using a DFR-specific enzymatic assay. The ScFv-coding region, provided with the appropriate expression signals, will be transformed to *P. hybrida* using the *Agrobacterium* vector system. Transgenic plants will be regenerated and inhibition of the enzyme *in vivo* will be followed by the phenotype of the flower. The color pattern of the petals will reveal the efficiency, as well as the stability and uniformity of the inhibition. It will be interesting to compare the inhibition by the "antibody" approach with that by anti-sense RNA.

T 511 ISOLATION OF ANTIBODY Fv-DNA FROM VARIOUS HYBRIDOMAS WITH A SIMPLE SET OF PRIMERS, Stefan Dübel, Frank Breitling, Mona Zewe, Patrick Fuchs, Stefanie Gotter, Martin Welschhoff, Gerd Moldenhauer, Melvyn Little, German Cancer Research Center, FSP4/0445, Im Neuenheimer Feld 506, 69120 Heidelberg, Germany.

To facilitate the isolation of antibody Fv-DNA sequences from murine hybridoma cell lines, we have established a PCR procedure requiring only a small number of primers. The sense primers homologous to DNA coding for the first framework sequences were designed to hybridize to all the known antibody sequences under conditions that permit a high number of mismatches. The antisense primers were homologous to DNA coding for the beginning of the constant regions of the γ,μ and κ chains. Restriction sites introduced by the primers enable the DNA to be cloned into bacterial expression vectors. Only two sense V_H primers and two antisense V_L primers paired with one backward primer for γ,μ and light chains, repectively, were necessary for the amplification of Fv-DNA from a total of nineteen rodent cell lines that we have so far worked with. These consisted of fourteen mouse cell lines and five rat cell lines, including one mouse hybridoma producing IgM. This procedure will therefore probably be sufficient to isolate the Fv-DNA from most mouse cell lines and possibly also from most rat cell lines.

T 512 CLONING AND EXPRESSION OF ANTIBODY FRAGMENTS FOR CONSTRUCTION OF A RECOMBINANT IMMUNOTOXIN. Rosanne Dunn[1], Peter Hudson[2], Glenn Lilley[2] and Robert L. Raison[1]. [1]ImmunobiologyUnit, University of Technology, Sydney, Australia, and [2]CSIRO Division of Biomolecular Engineering, Parkville, Victoria, Australia.

Recent studies have shown that genetically engineered antibody fragments have great potential in immunotherapy. Murine derived monoclonal antibodies (mAbs) directed against specific tumour markers generally produce a human anti-mouse response which destroys the antibody and results in a reduced therapeutic response. Antibody fragments which retain antigen binding specificity, such as Fv (VH + VL),single chain Fv (scFv), single domain antibody (VH) and Fab, are less immunogenic and can be linked to cytotoxic agents using recombinant technology. We have used a PCR based strategy to isolate the genes encoding VH and VL from a mab (K121) which binds to human free kappa light chains and recognises a specific epitope (KMA) expressed on the surface of human myeloma and lymphoma cells. A scFv was constructed by linking VH and VL genes with an oligonucleotide encoding a flexible hydrophilic peptide.The VH and scFv gene fragments were ligated into the expression vector pPOW, and the constructs sequenced and expressed in *E.coli* cell lines TG-1 and TOPP2. Expression of the foreign proteins was monitored by Western blot using a monoclonal antibody against a flag peptide encoded at the carboxy terminal region of the gene fragment. Antibody fragments were isolated from the periplasmic fraction of the bacterial lysate and purified using anti-flag affinity chromatography and antibody activity assessed by ELISA. The scFv gene derived from mAb K121 is being used to construct a recombinant immunotoxin by fusion with the gene encoding melittin, a membrane lytic peptide derived from bee venom.

T 513 *RECOMBINANT ANTIBODY AGAINST THE ERYTHROCYTE ANTIGEN, RHESUS D.* Morten Dziegiel, Leif Kofoed Nielsen, Peter Sejer Andersen and Jan Engberg, *Blodbanken*, Department of Clinical Immunology, University Hospital, Blegdamsvej 9, DK-2100 Copenhagen and *Department of Biology*, The Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen, DENMARK.

Genes encoding variable regions were produced from hybridoma cells by the polymerase chain reaction (PCR). They were subsequently cloned into a vector containing the CH1 gene in frame with *g3* which encodes the surface protein p3 of the filamentous phage. This results in a VH-CH1-p3 fusion protein which as an additional feature harbours a trypsin cleavage site between CH1 and p3. The gene encoding the light chain was subsequently cloned into the same vector and phages displaying Fabs were produced.

Despite the monoclonal origin of the hybridoma cells we performed several rounds of affinity selection of phage on intact erythrocytes. Each round consisted of a primary incubation with Rhesus D negative erythrocytes, a secondary incubation of the resulting supernatant with Rhesus D positive erythrocytes, vigorous washing, elution of adhering phages by treatment with trypsin, and eventually infection with eluted phages to produce a phage stock for the next round. As a measure of non-specific adsorption to Rhesus D positive erythrocytes a parallel secondary incubation was carried out with Rhesus D negative erythrocytes. The ratio between numbers of phages eluted from Rhesus positive and Rhesus negative adsorption increased with each round. This was however not reflected in the percentage of clones binding specifically to Rhesus D antigen which remained unchanged from the second to the third round (3/12 and 2/10, respectively). Restriction fragment analysis indicated that all the binding clones were identical. The ability to agglutinate enzyme-treated erythrocytes without a secondary antibody indicates that Fabs do associate which results in valencies above one.

T 514 IMMUNOGLOBULIN FOLDING STABILITY GENETICALLY SCREENED IN *E. COLI* AND CONSTRUCTION OF A DISULFIDE-FREE VARIABLE DOMAIN

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REI_V, the variable domain of a κ light chain and a series of variants (single amino acid replacements) were produced in *E. coli* and purified as described [1]. Conformational stabilities of these proteins positively correlate with the respective yields (most plausibly because of differential protease-resistence).

A fusion protein consisting of the aminoterminal part of ToxR of *Vibrio cholerae* [2] and REI_V is synthesized in *E. coli* as an integral membrane protein with the immunoglobulin part located in the periplasmic compartment and the DNA-binding domain of ToxR facing the cytoplasm. In accord with the prevalent model of ToxR mechanism of action [3], dimerization of the periplasmic domain of the fusion protein was found necessary and sufficient for stimulation of transcription from the *ctx* promoter. Since folding stability controls REI_V steady-state concentration (see above), it was possible to derive a characteristic transcriptional signal in *E. coli* from the folding stability of each of the variants fused to ToxR.

A REI_V variant with particularly high folding stability was constructed. Amino acid replacements which abolish the intra-domain disulfide bond were tolerated by this variant without collapse of the ordered fold.

References

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T 515 SCREENING OF scFv LIBRARIES BY DISPLAY ON E.COLI SURFACE AND FLUORECENCE ACTIVATED CELL SORTING

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We have constructed a unique gene fusion system for targeting and anchoring normally soluble proteins on the external surface of *Escherichia coli* (Francisco, J.A., et al. PNAS **89**: 2713 1992). Briefly, for surface expression the gene of interest is fused in frame to the 3' of a sequence encoding an Lpp-OmpA(46-159) hybrid. Proteins expressed as Lpp-OmpA(46-159) fusions, are active and are almost 100% accessible on the *E.coli* surface when the cells are grown at 24 °C. A single chain Fv antibody fragment specific for digoxin was constructed by PCR amplification of the heavy and light chains from two separate hybridomas and expressed as a fusion to Lpp-OmpA(46-159). The fusion protein was expressed at a high level ($5x10^4$ molecules per cell) and was accessible on the cell surface as determined by a immunofluorescence microscopy, whole cell ELISA and flow cytometry. The surface displayed scFv bound to a digoxin-fluorescein conjugate (dig-FITC) with an apparent affinity of 10^{-8} M which is similar to the soluble scFv and the parent monoclonals. We showed that cells displaying a scFv on their surface can be distinguished from background *E.coli* by fluorescence activated cell sorting (FACS). In particular, cells expressing Lpp-OmpA(46-159)-scFv(digoxin) could be enriched from a 100,000 fold excess of background cells by two rounds of cell sorting and regrowth of the positive populations (Francisco et al. PNAS in press). Screening of antibody libraries displayed on the *E.coli* surface by FACS could offer several advanatges over phage display such as selection of antibodies solely on the basis of affinity for hapten in solution, lack of avidity effects and quantitative recovery of positive clones. The screening of both repertoir and CDR 3 randomization libraries by this technology will be discussed.

T 516 CD3sFv FUSION PROTEINS TO STUDY THE CORRELATION BETWEEN CD3/TCR INTERNALIZATION AND STIMULATION VERSUS INHIBITION OF T CELL ACTIVATION, Lisa K. Gilliland*, Nancy A. Norris*, Dale Yelton*, Martha S. Hayden#, Peter S. Linsley*, and Jeffrey A. Ledbetter*, *Dept. of Autoimmunity and Transplantation, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA 98121 and #Johnson and Johnson Biotech Center, San Diego, CA 92121.

The variable regions of the anti-human CD3 mAb G19-4 were cloned with an intervening $(Gly_4Ser)_3$ linker and expressed in COS cells as an sFv alone or with one of several affinity "tails". The tails were used to purify the fusion proteins and to study T cell activation by the different proteins when cross-linked. They include *(a)* two peptide tails (one is recognized by a monoclonal, the other forms a high affinity association with streptavidin), *(b)* the hinge-CH2-CH3 of human IgG1 mutated in the hinge region to favor monomeric protein expression, and *(c)* an aglycosylated form of the monomeric hinge-CH2-CH3 that has been reported to exhibit reduced FcR binding. These proteins specifically bind CD3 and induce signal transduction (measured as intracellular calcium flux and tyrosine phosphorylation). The proteins synergize with PMA to induce T cell proliferation but show variable effects on T cell proliferation in conjunction with a costimulatory signal. For example, the tail-less CD3sFv or G19-4 Fab fragments are highly mitogenic with immobilized anti-CD28; however, CD3sFv with a monomeric hinge-CH2-CH3 tail inhibits proliferiation of purified T cells under these conditions. We are currently examining the kinetics of CD3 modulation induced by the CD3sFv fusion proteins to determine whether differences in CD3 internalization can account for the stimulatory or inhibitory effects of these proteins on T cell activation. We have also generated a comparable panel of fusion proteins that bind to murine CD3 to test the ability of these proteins to activate or suppress the immune system in vivo.

T 517 COLICLONAL ANTIBODIES FOR THE DETECTION OF PLANT PATHOGENS, Remko A. Griep, Charlotte van Twisk and Arjen Schots, Laboratory for Monoclonal Antibodies, P.O. Box 9060, 6700 GW Wageningen, The Netherlands.

Detection of plant pathogens is becoming increasingly important for the certification of plants, seeds and planting materials. The main reason is the restriction of the use of pesticides, because of environmental concerns. The detection systems to be developed, should be highly specific and sensitive so the pathogen can be detected in an early stage of infection. Most agricultural inspection services use at present (monoclonal) antibody based assays, like ELISAs, because they can be routinely applied to process large numbers of samples. The need for specific and sensitive assays resulted in the use of many high affinity monoclonal antibodies (MAbs). However, it remained difficult to raise such MAbs since a lot of cross reactions were observed with extracts from healthy plants or with closely related nonpathogenic species.

A system was developed wherein antibody genes of antigen specific B-cells were expressed as scFv or Fab fragments in *E. coli*. B-cells were obtained from the spleen of an immunized mouse. Prior to selection the population was depleted of B-cells producing cross reacting antibodies. From the remaining population antigen specific B-cells were selected by panning or cell sorting using a flow cytometer. The antibody genes of selected B-cells were cloned and expressed in *E. coli* starting from single cells or using "in cell PCR".

This system has been applied to the selection of antibodies specific for beet necrotic yellow vein virus (BNYVV) and *Erwinia chrysanthemi*, pathogens of, respectively, sugar beet and potato. The advantages of this system are the avoidance of the inefficient cell fusion process, necessary for the production of hybridomas; the fact that the lectin binding Fc fragment is no longer present; and the maintenance of original pairings of the heavy and light chains, yielding high affinity antibodies.

T 518 CHARACTERIZATION OF A HUMAN ANTIBODY AGAINST THE CYTOMEGALOVIRUS PROTEIN ICP36 EXPRESSED IN E.COLI, BHK AND SF21 CELLS

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Human cytomegalovirus (HCMV) an ubiquitous herpesvirus mainly causes subclinical infections in humans. In the immunocomprimised host, however, it is the cause of severe and not infrequently fatal disease. More detailed knowledge of antigenic structures recognized by the human immune system and reproducible production of defined human monoclonal antibodies should help to improve diagnosis and therapy of human cytomegalovirus infections. The genes encoding the anti CMV antibody A3C5 were isolated, sequenced, and expressed in E.coli, mammalian cells (BHK), and insect cells (SF21). The antigenic determinant recognized by A3C5 had been localized to a linear epitope including amino acids 281 to 286 of the DNA binding proteins ICP 36 of human cytomegalovirus AD169. Due to the strong binding to the antibody the epitop proved to be a very good tag on proteins. Furthermore we compared physical and biological properties of purified Fab-Fragments and whole antibody expressed in E.coli, BHK, Sf21 and cells with antibody derived from the originall B-cell clone. A computer model of the antibody by homology search for similar CDR loops should help to understand more about the antibody-antigen complex.

T 519 ESTABLISHING A SCREEN FOR ANTIBODY/HAPTEN INTERACTION BASED ON *E. COLI* GENETICS

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In *Vibrio cholerae*, the toxin genes *ctxAB* are under control of gene product ToxR, a protein integrated in the cytoplasmic membrane. In response to environmental signals, the periplasmic domain of ToxR forms a homodimer. This, in turn, tethers together the respective cytoplasmic domains, which can then bind to the *ctx* regulatory region, and stimulate transcription [1].

The *toxR/ctx* gene control system was moved to *E. coli* essentially as described [2] and the periplasmic ToxR domain was replaced by F_v, single-chain F_v and F_{ab} fragments of an antibody with binding selectivity for a low molecular weight hapten. Bivalent haptens were synthesized and tested as agents to mediate dimerization of the respective ToxR/immunoglobulin fusion proteins, thus stimulating transcription of a reporter gene placed under *ctx* control.

References

[1] DiRita, V. J.(1992) *Mol. Microbiol.* **6**, 451-458.
[2] Miller, V. L. & Mekalanos J. J. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3471-3475.

T 520 FOUR SUBCLASSES OF SWINE IgG IDENTIFIED FROM THE cDNA SEQUENCES OF A SINGLE ANIMAL,

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The sequences of more than 40 partial and complete swine Cγ cDNAs obtained by PCR cloning of first strand cDNA, and from a cDNA expression library, all from a single animal, have been compared. These sequences appear to represent four different IgG subclasses, with one expressed in two apparent allelic forms. Based on the non-conserved amino acid differences throughout the CH2 and CH3 domains, the multiple sequence alignment analyses indicated two major clusters, one composed of the subclasses IgG1 and IgG3 and the other composed of IgG2 and IgG4. Sequence comparisons of these putative subclasses and those of human and mouse IgG subclasses indicates that these swine IgG subclasses display the lowest intersubclass heterogeneity. The major subclass differences are located in the hinge and C-terminal portion of the Cγ3, similar in this regard to the differences among mouse subclasses. However differences in upper hinge length which correlate with segmental flexibility were not seen. All subclasses have identical middle hinge segments with sequences which suggest the presence of three interheavy chain disulfide bridges. The light chain appears to join to the CH1 domain as is the case for most IgGs in other species. Partial sequence analyses of PCR-amplified genomic DNA accounted for the two major groups of cDNAs; however a particular genomic sequence was identified that was different from any of the expressed sequences suggesting the existence of an additional Cγ gene. This was confirmed in genomic blots which suggest up to eight copies of Cγ. Collectively, these data suggest that swine have the largest number and most similar array of Cγ genes yet described for any species.

The data show high similarity between the swine and human IgG subclasses to strengthening the case for their use as models in immunology.

T 521 GENETICALLY ENGINEERED CONSTANT REGION ALTERATIONS OF A CLINICALLY RELEVANT ADENOCARCINOMA REACTIVE HUMAN IgG_3 MONOCLONAL ANTIBODY,

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88BV59 is a pan-adenocarcinoma reactive human IgG_3 κ antibody currently in Phase III registration trials for the radioimmunodetection of colorectal carcinoma. We cloned the expressed complete heavy and light chain cDNAs from 88BV59 and engineered them into pSV_2-neo and pSV_2-gpt derived shuttle vectors. Upon electroporating the light chain cDNA based vector, human-murine heteromyeloma cell lines were identified which secreted free κ light chain. We then precisely deleted the CH_2 domain of the γ_3 constant region by site-directed mutagenesis. Transfection of this ΔCH_2 cDNA based vector into the κ light chain producers resulted in cell lines secreting H_2L_2 dimers. In a separate experiment, the γ_3 constant region was replaced by a γ_1 cDNA in a manner similar to that described by Coloma, et al., J. Immunol. Methods *152*:89 (1992). Electroporation of the γ_1-cDNA based vector into the κ secreting cell lines resulted in the secretion of intact H_2L_2 dimers. ELISA and immunohistochemical analysis on human colon tumor xenografts indicate that both recombinant molecules bind their cognate antigen in a manner very similar to their IgG_3 parent. The shorter ΔCH_2 heavy chain with its abrogated effector functions may have more favorable pharmacokinetics than the intact γ_3 for radioimmunoscintigraphy. The IgG_1 isotype switch variant may have immunotherapeutical applications. The recombinant human antibodies have been scaled up in proprietary hollow fiber bioreactors and are currently undergoing preclinical evaluation.

T 522 LIPID-TAGGED ANTIBODIES FOR IMMOBILIZATION ONTO LIPID BILAYERS, Marja-Leena Laukkanen and Kari Keinänen, VTT Biotechnical Laboratory, P.O.Box 202, SF-02151 Espoo, Finland

We have studied possibilities to anchor antibody fragments onto lipid bilayers via hydrophobic tags for immunodiagnostic and other applications. Previously we reported that a bacterially produced anti-2-phenyloxazolone single-chain Fv antibody fragment (Ox scFv, Takkinen *et al.*, 1991) can be converted by genetic engineering into a membrane-anchored protein by fusion with the major lipoprotein (lpp) of *E. coli* (Laukkanen *et al.*, 1993). The resulting antibody fragment (Ox lpp-scFv) contains an N-terminal covalently- bound lipid tag and is stably associated with bacterial cell envelope and displays antigen-binding activity in membrane-bound as well as detergent-solubilized form.
In order to purify the fusion protein by immobilized metal affinity chromatography (IMAC), a lipid-tagged antibody with poly-His tail (Ox lpp-scFv-H6) was constructed. The expression of the Ox lpp-scFv-H6 in *E. coli* resulted in the production of a 30 kDa fatty acid modified protein with antigen-binding profile similar to parental Ox lpp-scFv. Purification by IMAC followed by hapten affinity chromatography yielded a highly purified lipid-tagged antibody which was reconstituted into liposomes by detergent dialysis. Liposomes carrying the antibody show specific antigen-binding activity measured as by ELISA and by real-time biospecific interaction analysis using BIAcore.
Lipid-tagged antibodies may find use in immunoliposome, vaccine and biosensor technology.

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T 523 USE OF ALANINE SCANNING MUTAGENESIS TO IMPROVE THE AFFINITY OF AN ANTI gp120 (HIV) ANTIBODY
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Antibodies that recognize V3 loop peptides of HIV gp120 have been shown to neutralize HIV*in vitro* and *in vivo*. It has been demonstrated that the antibody-antigen off-rate is an important parameter in neutralization, therefore the ability to decrease the off-rate of an antibody through mutagenesis is likely to increase its therapeutic potential. We isolated several mutants of a single chain Fv antibody (clone P5Q) with reduced off-rates to V3 loop peptides. Critical residues of the VH CDR3 region were identified by alanine substitution mutagenesis (alanine scanning). Four classes of affinities, as determined by BIAcore analysis, were observed upon replacement of alanine at each of the 27 amino acid positions of CDR3: i) increased binding (2), ii) decreased binding (5), iii) no binding (6), and iv) no change (14) relative to P5Q. Positions that resulted in increased or reduced binding (classes i and ii), operationally defined as critical, were candidates for further study.

The two class i positions (improved off-rates) were subsequently randomized to all amino acids and optimal solutions determined. In one case the optimized improvement is observed with glutamic acid (2.8 fold improvement relative to P5Q). Smaller improvements are observed with other polar or negatively charged amino acids, while binding was eliminated with hydrophobic residue substitutions. In the second case optimized improvement was observed with tryptophan (4.7 fold), and measurable improvements were observed with several hydrophobic residues. Thus the method is not limited to just one kind of amino acid substitution. Additional studies on one of these positions demonstrates that the improvement is observed with several gp120 V3 loop peptide variants.

To date, one member of class ii (decreased binding) has been randomized. In contrast to the class i residues described above, the aspartic acid which appears at this position in P5Q is the optimal residue. To determine whether or not improvements are additive, scFv derivatives which contain optimal amino acids at some or all of these positions are being evaluated.

T 524 CONSTRUCTION OF SINGLE-CHAIN ANTIBODY FRAGMENTS AND EVALUATION IN WHOLE-BLOOD DIAGNOSTIC KITS, Glenn G. Lilley, Greg Coia, Olan Dolezal, Carmel Hillyard[1], Dennis Rylatt[1] and Peter Hudson, CSIRO Division of Biomolecular Engineering, 343 Royal Parade, Parkville, Australia, 3052, and [1]AGEN Biomedical Limited, P.O. Box 391, Acacia Ridge, Australia, 4110.

We have designed, constructed and expressed scFv molecules that can replace Fab fragments as red blood cell agglutination reagents. The new technology has enabled the construction of antibody-like molecules with multiple functionality or altered or even increased specificity.
The SimpliRED diagnostic kit for HIV-1 (AGEN Biomedical Ltd., Australia) incorporates as the active reagent, a bifunctional, antibody-based molecule which is constructed by protein chemical techniques from the Fab portion of a mouse monoclonal antibody and a synthetic peptide epitope. The Fab domain recognises the glycoprotein, glycophorin-A found on the surface of human erythrocytes and the linked peptide epitope is in turn recognised by antibodies in the sera of individuals who have contacted and raised an immune response to HIV. The addition of the reagent to the blood of these patients causes rapid red cell agglutination.
The monoclonal antibody which forms the basis of the SimpliRED kit reagent has been cloned and the functional variable domains have been sub-cloned into *E.coli* expression vectors in the form of an scFv molecule fused to the FLAG octapeptide epitope or alternatively to HIV-1 epitopes (gp41, gp120 and p24 fragments). The products expressed in *E.coli* are recognised in Western blots by monoclonal antibodies directed against the C-terminal epitopes. The recombinant fusion protein of scFv and FLAG epitope can mimic the commercial reagent in agglutination assays. Research is directed towards the improvement of the expression system and purification methods for the use of the recombinant scFv reagents in robust diagnostic kits.

T 525 **IDIOTYPE MAPPING OF ANTI-DNA Fab FRAGMENTS EXPRESSED IN E. COLI,**

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Patients with lupus erythematosus (SLE) characteristically produce antibodies to (ds)DNA. The pathogenic importance of these antibodies is suggested by their fluctuation with disease activity. Idiotypic analysis of anti-DNA antibodies have been informative regarding the structural basis for DNA binding and for pathogenicity. Serum titter of 3I and F4 idiotypes, identified by monoclonal antibodies, correlate with serum levels of anti-dsDNA activity in SLE patients. This study has focused on the 2A4 antibody which possesses high affinity anti-dsDNA activity and expresses both the 3I and F4 idiotypes, located on the light and heavy chains respectively. Using PCR we have amplified the 2A4 C_H1,V_H4 and C_k,V_kI genes, 700bp each, and insert them separately or together into the pCOMB-3 plasmid vector. Several E.Coli clones expressing light chain, heavy chain and Fab fragments were obtained. High level expression can be seen both in bacteria cells and supernatants. Deletions and directed mutagenesis are been performed now in order to identify the exact idiotypes sequences. This antibody engineering approach provides a means for idiotype analysis and may identify the correlates to idiotypic and antigenic specificity and pathogenicity.

T 526 Construction of Single Chain Fv and Bi-Specific Antibody Fragments Directed Against p185 c-erb2 and EGFR

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p185neu is a tyrosine kinase cell surface receptor originally discovered in a transfection/cell proliferation assay of DNA from mutagen treated rats. The human homologue of p185neu is p185c-erbB2 which is over-expressed in several adenocarcinomas of secretory epithelial origin including breast, colon, ovary, and pancreas. Previous work from our laboratory has demonstrated that monoclonal antibody 7.16.4 binds to p185neu and specifically inhibits growth of neu transfected NIH3T3 cells in soft agar and invivo. Our results presented here show that mAb7.16.4 also binds to the human form of the receptor and causes tumor cell growth inhibition of cells over-expressing p185c-erbB2. In both cases, p185neu and p185c-erbB2, mAb 7.16.4 causes down-modulation of the receptor and the anti-tumor effects are independent of other cell mediators. The observations that 7.16.4 binds specifically to tumor cells over-expressing p185c-erbB2 and binding alone is sufficient for tumor growth inhibition make it highly desirable for construction of a single chain Fv fragment (scFv) for potential use in diagnosis and treatment of human cancer patients. We have cloned and sequenced both the heavy and light chain cDNA of mAb7.16.4. Our strategy for constructing, expressing, and purifying a 7.16.4 scFV is presented.

Epidermal growth factor receptor (EGFR) expression is also highly associated with several tumor types. Breast cancer patients with tumor cells which are positive for EGFR expression and over-express p185c-erbB2 have a poor prognosis. Previous work in our laboratory and others has demonstrated that stimulated EGFR and p184neu form highly phosphorylated heterodimers . Additionally, we have previously reported a synergistic anti-tumor effect when cells expressing both EGFR and p185neu are treated with mAb 7.16.4 and an anti-EGFR mAb, 425. We have cloned the cDNA of 3-162-2, another monoclonal anti-EGFR antibody and its sequence is presented. By combining the heavy and light chain variable fragments of mAb 7.16.4 with mAb fragments of either 3-162-2 or 425, we are producing a bi-specific scFv that may have greater specificity and anti-tumor effects against cells expressing both receptors.

T 527 FUNCTIONAL CHARACTERISATION OF A HUMAN MONOCLONAL ANTI-HIV-1 NEUTRALISING ANTIBODY EXPRESSED AS IGG1 AND IGG3 IN CHO CELLS, Florian Rüker, Renate Predl, Alexandra Trkola, Martin Purtscher, Andrea Buchacher, Christa Tauer, Franz Steindl, Thomas Muster, Willi Steinfellner and Hermann Katinger, Institute of Applied Microbiology, University of Agriculture, Nußdorfer Lände 11, A - 1190 Vienna, Austria

We have isolated a HIV-1 specific human monoclonal antibody, 2F5, which recognizes a conserved epitope on gp41 and neutralizes a wide range of different HIV-1 isolates *in vitro* as assayed by inhibition of syncytia formation and p24 assay. 2F5 has an IgG3 heavy and a kappa light chain, and shows moderate complement activation and only weak ADCC in vitro. We have cloned and sequenced the genes coding for the heavy and the light chain of 2F5, and we have coexpressed these genes in CHO cells to yield recombinant 2F5. In an attempt to engineer an antibody with improved effector functions, we have recombined the variable region of the heavy chain of 2F5 with a constant region gene of the IgG1 class. This gene was coexpressed with the original 2F5 light chain in CHO cells. A comparison of the in vitro characteristics of these 3 variants of the same monoclonal antibody will be shown.

T 528 CLINICAL EXPERIENCE WITH CDP571, AN ENGINEERED ANTI-TNFα ANTIBODY. Sue Stephens, Spencer Emtage, Olivia Vetterlein, Dee Athwal, Lesley Chaplin, Mark, Sopwith, Mark Bodmer. Celltech Research, 216 Bath Road, Slough Berks, England.

TNFα has been implicated in the pathology of a number of diseases, including septic and haemorrhagic shock, allograft rejection, rheumatoid arthritis and inflammatory bowel disease. Monoclonal antibodies to TNFα have been shown to have a protective effect in models of these diseases and, more recently, clinical trials of a murine anti-TNF in man have demonstrated therapeutic benefit in septic shock.

We have developed a recombinant antibody (CDP571) in which murine CDRs are expressed in a human Eu framework with human γ4 heavy chain and κ light chain constant regions. Comparisons in the cynomolgus monkey with the murine parent molecule (CB0010) have shown a 2-3 fold increase in βt1/2 and a 90% reduction in induced immune response. Responses to the constant regions were almost completely eliminated and responses to the idiotype were also dramatically reduced. The antibody class showed the classic temporal pattern of IgM followed by IgG.

Phase I studies with this molecule in 24 healthy human volunteers have now been completed. The elimination half-life at the higher doses (2-10mg/kg) is approximately 2 weeks and CDP571 was still detectable in the circulation at 3 months in a proportion of individuals. Immune responses, where detectable, are low, peak at 2 weeks, decline thereafter and are directed entirely against the idiotype. Unlike in monkeys, the anti-CDP571 response is predominantly of the IgM class and there is only an occasional weak switch to IgG production.

This antibody should therefore prove suitable for use in both acute conditions such as septic shock and in more chronic indications such as rheumatoid arthritis where repeated therapy may be required. Trials in these indications are currently in progress.

T 529 IMPROVED METHODS FOR CLONING, EXPRESSION AND ANALYSIS OF RECOMBINANT ANTIBODIES, Mark A. Sullivan, Jane Malone and Lorie LaPierre, Clinical Diagnostics Division, Eastman Kodak Company, Rochester, NY 14650-2117 (716) 722-6134

Conventional methods for the production of recombinant antibodies in *E. coli* have involved PCR amplification of VH and VL domains, followed by restriction cleavage or splice overlap PCR to assemble scFv or Fab constructs for cloning. A variety of techniques have been utilized for analysis of expression and function of the antibodies. We have applied new methods which simplify the assembly and expression of these molecules. Uracil residues have been incorporated into the PCR primers so that the VH and VL PCR products can be directly assembled and cloned in one step after a short treatment with uracil glycosylase. Primers have been designed to construct either scFv or Fab fragments with this method.

A series of vectors have been developed for expression of either free protein or M13 gene III fusion proteins for display as phage antibodies. These vectors also include the Flag™ sequence to permit direct measurement of binding activity of the antibodies using a monoclonal anti-Flag antibody in a simple ELISA format. The Flag sequence can be located at either the amino- or carboxy-terminus of the proteins. We have demonstrated the usefulness of these vectors and methods by cloning and characterizing a series of mouse hybridomas directed against phenobarbital.

T 530 CHARACTERIZATION OF SINGLE-CHAIN ANTIBODIES WITH DIFFERENT LINKER PEPTIDES

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For many applications small antibody fragments consisting of only the variable domains of the antibody molecule are advantageous. We have produced in *Escherichia coli* 2-phenyloxazolone (phOx) binding single-chain antibodies (scFvs) in which the variable domain of the heavy chain (V_H) has been joined to the variable domain of the light chain (V_L) with a flexible linker peptide of cellobiohydrolase I (CBHI) from *Trichoderma reesei*[1]. As an approach to design an optimal linker peptide we have studied the effect of the linker length on the activity and stability of the phOx scFv. Several derivatives of the phOx scFv with shortened linker peptides were produced as active hapten binding fragments in the culture medium of *E.coli*. We report here the hapten binding properties of the different phOx scFv fragments analyzed with BIAcore.

[1]Takkinen et al. (1991) Protein Engineering 4, 837-841.

T 531 CONSTRUCTION OF RECOMBINANT ANTIBODIES AGAINST *PSEUDOMONAS AERUGINOSA* LIPOPOLYSACCHARIDE

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P. aeruginosa lung infections remain the leading cause of death among cystic fibrosis (CF) patients. Lipopolysaccharide (LPS) is a virulence factor that plays a central role in the pathogenesis of the disease. A variety of murine hybridomas have been developed in our laboratory against various portions of the LPS molecule. A-band LPS, a chemically distinct form of *P. aeruginosa* LPS has been found to be a common antigen among CF clinical isolates. It is therefore, the goal of this research to engineer an A-band specific monoclonal antibody from an existing hybridoma, N1F10, to neutralize *P. aeruginosa* in chronically infected CF lungs. Variable region sequences were amplified using the polymerase chain reaction and primers specific for the 5' conserved region of the gene and for the 3' μ and κ constant regions. Southern blot analysis of the PCR amplified products, using V_H family specific probes and a Cκ probe, confirmed their immunoglobulin gene origin. Using the M13-derived phagemid expression systems, pComb3 and pComb8, developed by Kang and co-workers, Scripps Institute, we have expressed the PCR amplified antibody genes coding for an Fab against A-band LPS. After induction of Fab expression with IPTG, *Escherichia coli* host cells were sonicated and the supernatants were used for the detection of recombinant Fab fragments. Analysis by ELISA confirmed that this Fab was specific for the original A-band LPS antigen. Sequencing results have revealed significant homologies to other anti-polysaccharide antibodies. Site-directed mutagenesis studies using PCR to improve the affinity of this Fab for LPS will be discussed.

T 532 PRODUCTION OF HUMAN RECOMBINANT ANTI-IgE ANTIBODIES,

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Circulating anti-IgE autoantibodies present in sera of patients with allergic disease have been shown to play a role in the regulation of IgE mediated reactions. In order to compare the frequency of autoantibodies and of antibodies against an exogenous antigen as well as to isolate human anti-IgE antibodies we generated Fab antibodies by repertoire cloning. Using a monovalent phage surface display expression system a combinatorial library of antibody heavy and light chains was constructed from peripheral blood mononuclear cells of an atopic donor immunised with tetanus toxoid. Screening of the library allowed the identification of a large number of phage displaying human Fab fragments (Phabs) against tetanus toxoid and IgE. Surprisingly, we found a high frequency of Phabs against particular IgE myelomas that was comparable to the frequency found for Phabs against tetanus toxoid. However, most of these Phabs were directed to different idiotypic determinants depending on the IgE myeloma used for the panning procedure. Nevertheless, two clones were found to have anti-isotypic specificity and were shown to react specifically with the CH2 domain of the IgE heavy chain.

Late Abstract

CHIMERIC E.coli L-ASPARAGINASE II RESISTANT TO INACTIVATION BY TRYPSIN WAS CONSTRUCTED BY GENETICALLY FUSING A SINGLE CHAIN ANTIBODY (DERIVED FROM A PROTECTIVE MAB) TO THE ENZYME MONOMER,

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For many pharmaceutical proteins, inactivation by enzymatic proteolysis makes maintaining appropriate serum levels problematic. We have demonstrated, that selected non-inhibitory monoclonal antibody (MAB) to the trypsin sensitive antileukemic agent E.coli L-asparaginase II, provides substantial (70%) and sustained protection against proteolytic inactivation by trypsin. Inactivation of L-asparaginase was found to be associated with a single cleavage adjacent to lysine-29, and the protective MAB prevented this trypsin cleavage. We proceeded and demonstrated this trypsin sensitive enzyme can be rendered trypsin resistant, by genetically fusing it's gene with that of a single chain antibody (SCA) that was derived from this MAB. Following refolding we reconstituted 10% of the expressed chimeric protein to fully active form (presumably tetrameric). The chimeric L-asparaginase retained 75% of its original activity upon exposure to trypsin, while the native unprotected L-asparaginase-control was totally inactivated. This new chimeric trypsin resistant L-asparaginase, is expected to have a longer half-life in circulation, resulting in greater clinical utility. Such new chimeric protein-drug could be enhanced further by "humanizing" the SCA prior to gene fusion.