## MELANOMA AND BIOLOGY OF THE NEURAL CREST

*Organizers: Michael Lotze, Margaret Tucker and Mark Israel*

February 1-8, 1992

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*Keynote Address (Joint)*

**K 001** GENETIC ANALYSIS OF TUMOR REJECTION ANTIGENS, Thierry Boon, Ludwig Institute for Cancer Research, Brussels unit and Cellular Genetics unit, Catholic University of Louvain, Brussels, Belgium

Most mouse tumors express antigens that are potential targets for T lymphocyte-mediated rejection responses. Some of these antigens are recognized by cytolytic T cells (CTL) and do not appear to be present on normal cells. Recent progress has made it possible to understand how these new antigens can arise on tumor cells. It is now generally accepted that the antigens recognized by CTL are small peptides of about nine residues presented in a groove formed by the external domains of class I molecules of the major histocompatibility complex (MHC). A major mechanism that generates new antigenic peptides is the occurrence of point mutation in active genes. These point mutations change one amino acid in the antigenic peptides. In some instances, this confers to the peptide encoded by the mutated region the ability to bind to MHC molecules. In other instances, the point mutations generate new epitopes on peptides that were already capable of binding. All these point mutations may lie at the origin of the very diverse antigens observed on tumors induced with chemical carcinogens. A second major mechanism for the generation of new antigenic peptides on tumor cells is the activation of genes that are silent in most normal adult cells. This has recently been observed for an antigen present on a mouse mastocytoma (1). Other mastocytoma tumors have been found wherein the same gene is activated and the same antigen is present.

For human tumors, many groups have observed that lymphocytes from tumor-bearing patients ("autologous lymphocytes") can often be restimulated with the tumor cells in vitro so as to produce CTL that lyse the tumor cells and do not lyse autologous control cells. Stable clonal lines of CTL have been obtained. They recognize a limited number of antigens (3-7) on the autologous tumor cell lines. Recently a gene coding for an antigen recognized by CTL on a melanoma cell line has been isolated. This gene belongs to a new gene family that includes at least three closely related genes. The gene is expressed on several melanomas. These tumors that express both the gene and HLA-A1 present the antigen recognized by the original CTL. These results indicate that tumor rejection antigens recognized by CTL may be shared by large numbers of tumors. They also suggest that patients whose tumor expresses these antigens could be identified rapidly by HLA typing and RNA analysis of a small tumor sample. These patients could receive precisely targeted immunotherapy.

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*Melanobiology*

**K 002** RECEPTOR TYROSINE KINASES IN NORMAL AND MALIGNANT MELANOCYTES, Ruth Halaban. Department of Dermatology, Yale University School of Medicine, New Haven, CT 06510.

A most striking difference between normal melanocytes and melanoma cells from advanced lesions *in vitro* is in the requirement for growth factors. Unlike melanoma cells, normal human melanocytes depend on exogenous growth factors that stimulate their proliferation in the presence of the synergistic factors dibutyryl cAMP (cyclic adenosine monophosphate) or TPA (12-*O*-tetradecanoyl phorbol-13-acetate). The melanocyte mitogenic peptides are certain members from the Fibroblast Growth Factor (FGF) family, and two unrelated ligands, Hepatocyte Growth Factor (HGF) and Mast Cell Growth Factor (MGF, the Kit-ligand) (reviewed in reference 1, 2). Two mutually synergistic melanocyte mitogens are bFGF and HGF (3). Activation of the receptors for FGFs, HGF and MGF (FGFR, Met and Kit, respectively, all transmembrane protein-tyrosine kinase) stimulates autophosphorylation and a cascade of phosphorylations and activation of other enzymes involved in signal transduction. A common intermediate for all three in human melanocytes is MAP-2 kinase/ERK (2, 3). Melanomas have acquired autonomy in part by the inappropriate production of bFGF, which constitutively activates the FGF-receptor. However, the expression of bFGF by itself is not sufficient to transform normal melanocytes to melanomas, suggesting the presence of at least one co-operating factor. This co-operating factor is not a constitutively active c-Met, the HGF receptor. Met is normal in melanomas, and melanomas do not express HGF. However, activation of c-Met may enhance dissemination of primary melanomas to ectopic sites, because HGF (known to be also a scatter factor), stimulates normal human melanocyte migration (3). Kit-kinase on the other hand, does not positively regulate melanoma cell proliferation because c-*kit* is frequently down-regulated in melanoma cells grown *in vitro* from advanced lesions (2). Because activation of Kit-kinase in normal melanocytes leads not only to proliferation but also to induction of differentiated functions, it is possible that extinction of c-Kit-kinase signal transduction contributes to the dedifferentiated phenotype of many melanomas.

Animal models give further evidence for the importance of receptor tyrosine kinases in normal melanocyte proliferation and transformation to melanomas. Normal function of c-Kit was shown to be important for pigment cell viability and proliferation through genetic analyses of mice with mutations at the dominant white spotting (*W*) and steel (*Sl*) loci, encoding *kit* and its ligand MGF, respectively. *In vivo* transformation of mouse melanocytes to melanoma, due to constitutive expression of a transmembrane tyrosine kinase related to c-*kit*, the oncogene *ret*, was recently demonstrated in transgenic mice. Finally, studies on a fish model, *Xiphophorus*, in which melanoma is inherited, have shown that the dominant tumor inducing gene, *Tu*, encodes an EGF-receptor related tyrosine kinase which is expressed only in the melanomas and not in normal tissues (reviewed in reference 1). Taken together, the results suggest that normal melanocyte proliferation and the uncontrolled growth of melanomas is dependent, in large part, on the proper control of receptors with tyrosine kinase activity.

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**K 003** UNDIFFERENTIATED KERATINOCYTES CONTROL GROWTH, MORPHOLOGY, AND ANTIGEN EXPRESSION OF NORMAL KERATINOCYTES THROUGH CELL-CELL CONTACT, Meenhard Herlyn, and Istvan Valyi-Nagy, The Wistar Institute of Anatomy and Biology, Program of Experimental Therapeutics, 36th and Spruce Streets, Philadelphia, PA

Growth and phenotype of normal human melanocytes is highly affected by undifferentiated keratinocytes. Melanocyte growth was supported neither by conditioned medium of keratinocyte cultures nor by co-cultures of melanocytes with keratinocytes when separated by microporous membranes. In contrast, in monolayer co-cultures allowing physical contact between the two cell types, both cell types proliferated, but their ratio remained constant over a 14-day period. In three-dimensional epidermal reconstructs, most melanocytes were found singly in the basal layer and they communicated with undifferentiated keratinocytes. Conversely, melanocytes entrapped in the differentiated layers of epidermal reconstructs were not growth controlled and formed multiple-cell colonies. These clustering cells did not communicate with adjacent keratinocytes. The pattern of antigen expression of melanocytes growing in monolayer co-cultures and in the basal layer of epidermal reconstructs was indistinguishable from that in skin. Melanocytes, on the other hand, proliferating in pure monolayer cultures or as colonies within differentiated layers of epidermal reconstructs expressed several melanoma-associated antigens such as O-acetylated ganglioside GD3 or a 120 kD protein detected by monoclonal antibody 77.1. These results suggest that undifferentiated but not differentiated keratinocytes control growth, morphology, and antigen expression of melanocytes through direct cell-cell contact. The mechanisms of this regulation remains to be investigated.

*The Epidemiology and Genetics of Melanoma*

**K 004** CHANGES IN GENE EXPRESSION (JUN, PROTEIN KINASE C, AND RETINOIC ACID RECEPTOR FAMILIES) DURING HUMAN MELANOCYTE PROGRESSION TO MALIGNANCY, Frank L. Meyskens, Jr., Douglas Yamanishi, Helen Ross, Julie Buckmeier. Department of Internal Medicine and Clinical Cancer Center, University of California Irvine, Irvine, California 92715.

The evolution of human melanoma is accompanied by characteristic clonal cytogenetic abnormalities, antigenic changes, and growth factor alterations. Identification of specific oncogene alterations in the formation of melanoma has been few, with activation of ras in late cultured cells occurring in 25% of cases. One of the unique culture requirements for melanocyte growth is TPA; paradoxically this chemical also inhibits melanoma cell growth. We therefore performed a series of studies on the role of protein kinase C in melanoma biology. Three of the PKC isotypes ($\alpha$, $\beta$, $\epsilon$) were constitutively expressed in neonatal melanocytes. In melanoma cells the expression of PKC $\alpha$ and $\epsilon$ isotypes was altered and PKC $\beta$ RNA transcripts were not expressed. TPA did not induce $\beta$-transcripts and Southern analysis after digestion with four different restriction enzymes did not show rearrangement or deletion of the PKC-$\beta$ gene. These interesting results led us also to examine changes in TPA induced oncogenes. Uniformly high transcript levels of jun-B, and c-fos RNA were observed in melanocytes cultivated in complete medium. In comparison to melanocytes, metastatic melanoma cells expressed an increase in jun-B fos RNA transcripts and a decrease in c-jun transcripts. Broadly interpreted, these data suggest a dysregulation of these genes in melanoma cells. In the past we have done a number of studies with retinoids and melanoma cells. We have recently found marked sensitivity of melanocytes to $\beta$-transretinoic acid ($ID_{50}$ 1-4 $\mu$g/ml). This is of considerable interest since it is known that c-jun protein interacts with nuclear retinoic acid receptor (RAR) function and expression at the RNA level. All three transcripts ($\alpha$, $\beta$, $\gamma$) of RAR were expressed in neonatal melanocytes. $\alpha$ and $\gamma$ were expressed in all four melanomas examined but $\beta$ was expressed in only two. Two of the melanoma cell lines did not express RAR-$\beta$ and RAR-$\beta$ transcripts were not induced by RA. A number of ongoing studies involving the growth effect and expression of RAR in melanoma cells measure the effect of biochemical modulators (steroid hormones, cytokines, and protein kinase C and phosphatase activators) alone and in combination with RA.

**K 005** GENOMIC AND BIOLOGIC STRATEGIES FOR IDENTIFYING A GENE ON CHROMOSOME 6 IMPLICATED IN MELANOMA TUMORIGENESIS J. Trent, Ph.D.[1,2], E. Meese, Ph.D.[1], X.Y Guan[3], P. Lagoda, Ph.D.[1], P. Meltzer, M.D., Ph.D.[1,3] Depts. of [1]Radiation Oncology, [2]Human Genetics & [3]Pediatrics, Univ. of Michigan, MSRBII/C560, Ann Arbor, MI 48109 & [4]Comm. on Genetics, Univ. of Arizona, Tucson, AZ 95724.

Previous cytogenetic studies have suggested that the long arm of chromosome 6 is frequently deleted in malignant melanoma. The possibility that this region encodes a tumor suppressor gene is supported by studies which demonstrate that introduction of a normal chromosome 6 suppresses the tumorigenicity of melanoma cell lines (Trent, *et al.*, *Science* 247:568, 1990). Studies aimed at identifying genes along chromosome 6 have been hampered because a limited number of probes to the long arm of chromosome 6 are presently available, and numerous additional probes will be required to generate a physical map of this region. Several different strategies have been taken in order to develop the genomic resources required for identifying this proposed suppressor gene. Among them have included the establishment of a somatic cell hybrid mapping panel which allows mapping of DNA markers into eight distinct physical regions of chromosome 6. Characterization of the hybrid panel members has been based upon cytogenetic, fluorescent *in situ* hybridization (using plasmid, cosmid, YAC and painting probes), and Southern blotting (using known reference probes). Utilizing this panel, 17 NotI boundary clones from a NotI-linking library were regionally assigned to the long arm of chromosome 6. The NotI sites likely indicate HTF-islands and coding sequences, accordingly, cross-hybridization of the linking clones to DNA from other species provided further evidence for transcribe sequences in 7/17 NotI clones. The development of this panel should facilitate the construction of a physical and genetic linkage map to assist in the identification of the proposed suppressor gene. In order to obtain additional region specific probes, microdisection of banded metaphase chromosomes has also been used to isolate chromatin from human chromosome 6q21-23. Chromosomal fragments from this region were dissected and pooled in collection solution containing proteinase-K. The DNA was then amplified by the polymerase chain reaction (PCR) using an Alu primer. The PCR products were directly cloned by a TA-cloning procedure using the pCR™ vector. Several hundred colonies were isolated and we have characterized more than 50 clones to date. Inserts for this library range in size from 160-1200 base pairs. Clones have been selected and hybridized against DNA from the previously mentioned somatic cell hybrid panel, with the results confirming their derivation from human chromosome 6 derived sequences. These results indicate that microcloning of specific chromosome bands will be a valuable tool in generating region specific libraries. Finally, concurrent biologic strategies are underway to more clearly delineate the region of 6 involved in the suppression of melanoma. The microcell-mediated chromosome transfer technique has been utilized to generate irradiation reduction hybrids for subsequent analyses of suppression. This procedure involves irradiation of microcells which contain a single human chromosome 6 (marked by the integration of psv$_2$neo) with lethal irradiation at 8,000 rads, and fusion to a nonirradiated host. A panel of deletion hybrids for chromosome 6 has been generated and are being systematically evaluated for the presence of chromosome 6 specific sequences, and subsequently for their biologic activity in functional assays for suppressor activity. Research supported in part by CA-29476 and CA-41183.

*Antigenes Defined By T Cells*

**K 006** CYTOLYTIC T LYMPHOCYTE RESPONSE TO AUTOLOGOUS MELANOMA: ROLE OF INTEGRINS AND ADHESION MOLECULES. Andrea Anichini[1], Roberta Mortarini[1], Saverio Alberti[2], Alberto Mantovani[2] and Giorgio Parmiani[1], [1]Division of Experimental Oncology D Istituto Nazionale Tumori, 20134 ,Milan, Italy and [2] Istituto Ricerche Farmacologiche Mario Negri, 20157, Milan, Italy.

The clonal analysis of cytolytic T cell (CTL) response to metastatic melanoma cells has revealed that intra-tumor heterogeneity for susceptibility to lysis by cloned CTL is a common feature among tumors isolated from a number of patients. In fact, by cloning tumor cells with the soft agarose technique and testing melanoma clones for lysis by a panel of autologous CTL clones, it is possible to find tumor clones which are killed by most T cell effectors and other tumor clones which show a low degree of susceptibility to cell-mediated lysis. The phenotypic analysis of melanoma cells with low susceptibility to lysis revealed that all these clones expressed lower levels of the adhesion molecule ICAM-1 and of B1 integrins in comparison to the tumor cells with high susceptibility to lysis. The differential expression of ICAM-1 on melanoma clones, however, did not affect conjugate formation, as indicated by adhesion assays, suggesting that absence of lysis by some CTL clones might be primarily due to absence of T cell receptor (TcR) triggering by the neoplastic target. In fact, TcR triggering by antibodies to the CD3 complex induced CTL-mediated lysis of all neoplastic clones. In addition, modulation of ICAM-1 levels by IFNγ could substantially increase, but not induce, tumor cell lysis only by those CTL clones acting in a TcR-dependent and MHC-restricted fashion. These data suggested that ICAM-1 up-regulation might boost the efficacy of an LFA-1-dependent signal, acting in synergy with TcR triggering, thus leading to increased target lysis only by specific anti-tumor CTL. In fact, inhibition assays by monoclonal antibodies to LFA-1 or to ICAM-1 indicated that the increase of lysis observed after ICAM-1 up-regulation could be inhibited to similar values by interfering with recognition of either LFA-1 on the effector or ICAM-1 on the target. The role of ICAM-1 in the differential susceptibility to lysis by tumor-specific CTL clones was confirmed by transfecting the ICAM-1 gene in a melanoma clone expressing low levels of endogenous ICAM-1. The transfected clone expressed higher levels of ICAM-1 and increased susceptibility to lysis only by tumor-specific T cell clones acting in a TcR-dependent fashion, thus confirming that the level of expression of this adhesion molecule can affect melanoma cell lysis mainly when TcR triggering by tumor-associated antigens takes place. Finally, the potential role of B1 integrins expressed on melanoma clones in the CTL-tumor interaction was investigated by inhibition assays.The results of these experiments indicated that monoclonal antibodies to at least three different extracellular matrix receptors expressing the α4, α5, and α6 subunits of B1 integrins could significantly reduce the CTL-mediated lysis of melanoma cells. In addition, inhibition of lysis was seen with either tumor-specific and -non specific CTL, i.e. independently from TcR triggering. These data suggest that increased levels of B1 integrins on some melanoma cells might contribute to the interaction with all autologous CTL clones.

**K 007** MOLECULAR ENGINEERING OF THE ANTITUMOR IMMUNE RESPONSE, Drew Pardoll[1], Paul Golumbek[1], Liz Jaffe[1], Hy Levitsky[1], Glen Dranoff[2], and Richard Mulligan[2], [1]The Johns Hopkins University School of Medicine, Baltimore, MD. 21205 and [2]Whitehead Institute, Boston, MA. 02139

One of the most critical questions in cancer immunology is why the immune system fails to eliminate tumors that arise do novo. Concentrating on the T cell-mediated immune response, we sought to determine whether this was due to an absence of CTL precursors capable of recognizing tumor-specific neoantigens or rather, a failure of the helper arm to produce the lymphokines that act as critcal second signals for CTL activation. We devised a novel strategy to answer this questions that involved engineering tumor cells by gene transfaction to produce helper lymphokines known to be important for CTL priming. The critical feature of this approach is that the helper lymphokine is produced only where the antigens are. We reasoned that these engineered tumors might effectively activate "latent" tumor specific CTL by bypassing a defective T helper arm. When cells from a spontaneously arising renal cell tumor were engineered to secrete large doses of Interleukin-4 (IL-4) locally, they were rejected in a predominantly T cell-independent manner. In addition, animals that rejected the IL-4 transfected tumors developed T cell-development systemic immunity against the parental tumor. This systemic immunity was tumor-specific and primarily mediated by CD+ T cells. The systemic immune response generated by this approach was strong enough to cure animals with small amounts of parental tumor established in the mice prior to injection of the genetically engineered tumor. Certain lymphokine combinations that generate potent CD8 dependent immune responses require CD4 at the priming stage. It therefore appears that endogenous tumor-specific T helper cells are being converted from a tolerant state to an activated state. This hypothesis is currently being tested using TCR-transgenic mice bearing a TCR specific for an MHC class II-restricted tumor antigen. Because these results provide a rationale for the use of lymphokine gene-transfected tumor cells as a modality for cancer therapy, we have begun to use amphotrophic retroviral vectors to transduce primary human renal and ovarian tumor explants. The results of these efforts will be discussed.

**K 008** RECOGNITION OF MELANOMA ASSOCIATED ANTIGENS (MAA) BY HUMAN TUMOR INFILTRATING LYMPHOCYTES (TIL), Suzanne L. Topalian, Sophia S. Hom, Yutaka Kawakami, Marie Mancini, Douglas J. Schwartzentruber, Rina Zakut, and Steven A. Rosenberg, Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

TIL can be grown selectively from single cell digests of human melanomas in the presence of IL-2. These cultures, when propagated for approximately 30 days or more, become predominantly $CD3^+$, $CD8^+$, HLA-$DR^+$ lymphocyte populations whose clonality is limited, as shown by analysis of TCR-ß gene rearrangements. In short term $^{51}Cr$ release assays, many melanoma TIL cultures can lyse fresh or cultured autologous melanoma cells, but not tumors of other histologies nor autologous PBL, cultured fibroblasts, or EBV transformed B cell lines. Lysis can be inhibited by mAb directed against MHC class I molecules (W6/32, anti-ß2 microglobulin) or the TCR (anti-CD3). These data suggest that $CD8^+$ TIL function as CTL in recognizing a putative MAA. Studies using melanoma TIL to lyse HLA-matched allogeneic melanomas have shown that commonly expressed MAA can be recognized by TIL in the context of a variety of HLA-A, B, and C molecules. Lysis of HLA-matched nonmelanoma tumors, or unmatched allogeneic melanomas, was not observed. Lysis of HLA-matched allogeneic melanomas could be inhibited by W6/32 and anti-CD3, providing further evidence for specific recognition of shared MAA. To detect MAA on tumors of any HLA phenotype, six cultured melanoma lines which do not naturally express the HLA-A2 molecule were transfected with the gene for HLA-A2.1, and transfected tumor clones were used as targets for lysis by HLA-A2 restricted melanoma-specific TIL. All 17 clones expressing HLA-A2.1, but none of 12 clones failing to express this restriction element, were lysed by TIL, suggesting that common MAA are expressed among melanoma patients regardless of HLA type. Specific recognition of MAA by $CD8^+$ or $CD4^+$ TIL can also be detected by measuring the release of GM-CSF, IFN-$\gamma$, and TNF-$\alpha$ from TIL cocultured with the appropriate tumor stimulators. Cytokine release is MHC restricted, as it can be inhibited by mAb directed against MHC class I or class II molecules (for $CD8^+$ or $CD4^+$ cells, respectively), and can be used to detect shared MAA on allogeneic tumors. In vivo, $^{111}In$ labeled TIL infused into melanoma patients with metastatic disease have been shown to traffic specifically to sites of disease but not to normal tissue; the potential roles of cytolysis and/or cytokine release in mediating tumor regressions are currently under investigation. Studies are also in progress to identify the gene encoding a MAA, using TIL lysis or cytokine production to detect transfection recipients expressing the molecule(s) of interest.

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## *Use of Antisense Molecules*

**K 009** ANALYSIS OF THE FIBROBLAST GROWTH FACTOR RECEPTOR IN NORMAL HUMAN MELANOCYTES AND MALIGNANT MELANOMAS, Dorothea Becker[1], and Meenhard Herlyn[2], [1]Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, [2]The Wistar Institute, Philadelphia, PA 19104.

Over the past several years, human malignant melanoma and its precursor lesions has emerged as an ideal system for the study of molecular events governing the process of tumor progression. One of the findings that emerged as part of these studies was the observation that proliferation of primary and metastatic melanomas are dependent upon the autocrine production of bFGF, whereas normal melanocytes fail to express the bFGF gene at detectable levels. In contrast, studies presented here demonstrate expression of the fibroblast growth factor receptor in both normal melanocytes and malignant melanomas as evidenced by the presence of a 3.5 kb mRNA transcript and detection of a 145 kd protein product in these cells. Antisense oligonucleotides targeted against the translation start site and a splice donor-acceptor site of human fibroblast growth receptor not only inhibit the proliferation of normal melanocytes and malignant melanomas but seem to cause dendrite formation and disruption of cell-cell adhesion. The fact that none of these changes are apparent upon inhibition of expression of the bFGF gene may suggest a role of the FGF receptor as an important mediator in cell-cell interactions in the melanocytic system.

K 010 TRANSCRIPTIONAL INHIBITORS AND DIRECT GENE TRANSFER INTO TUMORS IN VIVO, Gary J. Nabel, Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, MI

The aberrant growth of melanoma and other malignant tumors is caused by the inappropriate expression of genes which regulate cell proliferation. Such gene products have served as targets for anti-neoplastic agents. One approach to the modification of gene expression in vivo is to develop transcriptional inhibitors, which prevent the synthesis of specific genes. We have generated such inhibitors which interfere with transcriptional activators by mimicking the cis-acting regulatory sequences to which they normally bind. This method relies on the use of the double-stranded form of modified oligonucleotides (phosphorothioates) which mimic DNA binding sites. These transcriptional inhibitors can affect gene expression specifically and exert potent effects on the viral and cellular gene expression to inhibit cell growth. A second approach to the treatment of malignancy is to introduce recombinant genes directly in vivo to inhibit tumor cell growth. The expression of foreign histocompatibility (MHC) proteins in living organisms stimulates a potent immune response which mediates tissue rejection. Expression of a foreign MHC gene has been directed to malignant tumors in vivo in an effort to stimulate tumor rejection. By direct gene transfer, a murine class I H-$2K^s$ gene was introduced in vivo into subcutaneous tumors from the CT26 mouse colon adenocarcinoma (H-$2^d$). Expression of this foreign MHC gene within tumors induced a cytotoxic T cell response to H-$2K^s$ and, more importantly, to other antigens present on unmodified tumor cells. This immune response attenuated tumor growth or caused complete tumor regression in several animals. This approach has also been applied to murine malignant melanoma. Direct introduction of recombinant genes into tumor cells in vivo provides a novel and effective method for the immunotherapy of malignancy.

K 011 USE OF ANTISENSE TECHNOLOGY TO PROBE THE ROLE OF ONCOGENES IN THE REGULATION OF NEUROECTODERMAL GROWTH AND DIFFERENTIATION, Len Neckers, Angelo Rosolen and Luke Whitesell, Clinical Pharmacology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

Neuroectodermal tumors of childhood provide a unique opportunity to examine the role of genes potentially regulating neuronal growth and differentiation because many cell lines derived from these tumors are composed of at least two distinct morphologic cell types. These types display unique phenotypic characteristics and spontaneously interconvert *in vitro*. One variant, the epithelial like S cell, is adherent, displays contact-inhibited growth, is unable to clone effectively in soft agar, and is non-tumorigenic in animals. The second variant, the neuroblastic N cell, is only weakly adherent, grows without contact inhibition, clones in soft agar, and is tumorigenic in animals. The factors that regulate the spontaneous interconversion of these two morphologic types are unknown, as are the reasons why both morphologies co-exist in culture over many passages. We have utilized antisense technology to study the role of the NMYC gene in these phenomena. Both antisense phosphodiester 15-mers complimentary to the translation start site of NMYC mRNA, as well NMYC antisense-expressing episomal replicons, can successfully block NMYC protein expression in these cells. Our findings implicate NMYC in maintenance of the cellular heterogeneity characteristic of these cell lines *in vitro*. In this way, NMYC expression may supply a growth advantage to the culture as a whole. However, although monomorphic clones proliferate less rapidly than wild type cultures, NMYC suppression does not abrogate tumorigenicity *in vivo*. These results demonstrate the utility of antisense technology in studying the role of individual genes which regulate the complex biology of neuroectodermal cell lines.

*Cytokines and the Endocrine System (Joint)*

K 012 PATHOGENIC ROLE OF CYTOKINES IN AUTOIMMUNE ENDOCRINE DISEASES, Klaus Bendtzen, Laboratory of Medical Immunology TTA 7544, Rigshospitalet University Hospital, DK-2200 Copenhagen N, Denmark.

Many models have been advanced to account for the association of HLA types with autoimmune endocrine diseases. For example, aberrant expression of MHC class II molecules may facilitate a T cell-mediated attack against 'forbidden antigens' on endocrine cells. However, cytokines produced by MHC class II-bearing macrophages (MØ) may also contribute to endocrine disease (1-4). Thus, IL-1 suppresses insulin production *in vitro* through a direct cytotoxic effect on pancreatic β-cells. This effect is modulated by IRAP, a specific IL-1 receptor antagonist, suggesting the involvement of 'classical' IL-1 receptors on β-cells or other islet cells involved in β-cell damage. The toxicity afforded by IL-1 is related to disturbed glucose metabolism in β-cell mitochondria. Other cytokines such as TNFα, IL-6 and IFNγ also influence insulin production, especially in conjunction with IL-1, but these cytokines are generally not cytotoxic. Treatment of the spontaneously diabetic BB rat with antibodies to IFNγ prevents the disease (5), and drugs such as cyclosporin and fusidic acid interfere with IL-1-mediated effects on rodent β-cells and with the development of spontaneous diabetes in the BB rat. Other endocrine cells are susceptible to modulation by cytokines. For example, IL-1 suppresses, but does not kill, human thyroid cells.

A stimulatory effect on insulin and thyroglobulin production is observed at extremely low concentrations of IL-1 and of higher but *in vivo* attainable levels of IL-6. This suggests that cytokines may have important functions as **physiological** regulators of endocrine cells, for example during conditions of stress. A role of IL-6 is further supported by the fact that IL-6 is produced by glucose-stimulated rat islets and by secondary cultures of human thyrocytes, in particular after challenge with IL-1. Also, IL-6 causes similar damage to pancreatic β-cells as does IL-1.

Proposed models for a role of cytokines in insulin-dependent diabetes mellitus and, possibly, in other autoimmune endocrine diseases:

**Model** 1: Initial damage (virus?) affects the endocrine target cells themselves. Accumulated MØ attract T and B lymphocytes and NK cells. The MØ, and/or the MHC class II+ targets themselves, present antigen(s) to specifically reactive T cells releasing additional cytokines. IL-1 and IL-6, potentiated by TNFα and IFNγ, further damage the cells. Eventually, autoantibodies and immune complexes contribute to β-cell destruction. The specificity in this model is governed by the specifically reactive T lymphocytes and by the specificity of the infectious or toxic damage inflicted upon the target cells.

**Model** 2: The initial toxic, inflammatory or infectious reaction(s) take place in the vicinity of the target cells, which as 'innocent bystanders' become the victims of an immunological attack. A perivascular process and/or vasculitis in afferent blood vessels trigger the production by endothelial cells and smooth muscle cells of IL-1, TNFα and IL-6, which may accumulate at high, β-cell toxic levels in the islets. This initially nonspecific reaction eventually becomes immunologically specific resulting in accelerated β-cell destruction. This model predicts the existence of several 'etiological factors' in the course of events leading to diabetes mellitus, even in the same individual. It also explains the finding of MHC class II proteins on vascular endothelium in prediabetic BB rats and in recent-onset diabetes mellitus, even in or around islets without MØ invasion (see 2).

In both models, the magnitude of a T cell-mediated attack depends upon the MHC class II type of the individual, thus accounting for the MHC association of many autoimmune endocrine disorders. However, since the TNFα gene is polymorphic and located in the MHC region, the disease-associated HLA types may be linked to TNFα alleles coding for prolonged and/or pronounced secretion of this cytokine. Thus, activated peritoneal MØ from the spontaneously diabetic BB rat secrete more TNFα than cells from diabetes-resistant or normal rats (6). Also, MØ from HLA-DR2+ healthy individuals respond less vigorously with respect to TNFα production; this might explain the protection afforded by HLA-DR2 in diabetes mellitus (see 2).

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**K 013 NATURAL REGULATORS OF T-CELL LYMPHOKINE PRODUCTION IN VIVO,** Raymond A. Daynes, Tad A. Dowell, and Barbara A. Araneo, University of Utah School of Medicine, Salt Lake City, UT 84132.

The development of protective immunity *in vivo* requires T cells to receive, integrate, and respond appropriately to a variety of exogenous and endogenous signaling systems. Foreign antigen represents the main exogenous signal, conferring specificity to immune responses by promoting the activation of only those T cells bearing complimentary cell surface receptors. The consequences of T-cell activation *in vivo*, however, appear to be internally controlled through the activities of endogenous substances capable of regulating the functional potential of recirculating T cells. Many distinct types of naturally occurring molecules exist that can alter the functional properties of T cells. Cytokines, growth factors, other polypeptide hormones, arachidonic acid metabolites, and numerous steroid hormones, have all been thoroughly investigated for their ability to modify the biochemical behavior of T cells. Some of these agents exert their effects systemically (e.g., glucocorticoids and certain cytokines) while the actions of others (e.g., prostaglandins, growth factors, and some steroid hormones) are anatomically restricted. Such controlling mechanisms are quite important to the immune system. They provide it with an extensive degree of flexibility plus the capacity to compartmentalize specific effector responses to particular tissue microenvironments.

We have been investigating the roles played by certain species of steroid hormones that gain access to lymphoid tissues as a consequence of end-organ metabolism of inactive circulating precursors. Localized production of dehydroepiandrosterone (DHEA) from its circulating sulfated precursor (DHEAS), coupled with the enhancing influence of DHEA on activated T-cell IL-2 and γIFN production, provides a simple biochemical means to regulate the functional potential of T cells residing within tissue sites where this hormone is being effectively metabolized. Our studies also indicate that dihydrotestosterone and 1,25-dihydroxyvitamin $D_3$ play equally important roles in controlling the nature of T-cell responsiveness within particular lymphoid organs in the body. Therefore, alterations to the functional properties of T cells that accompany stress, trauma, or aging may, in part, be due to fluctuations in tissue localized and systemic steroid hormone levels.

The results of our very recent studies implicate platelet-derived growth factor (PDGF) as being an extremely potent modulator to the functional properties of T cells. Once again, these controlling influences only become manifest after cellular activation. T cells exposed to nanogram quantities of PDGF are able to markedly enhance their IL-2 production following activation, while simultaneously depressing the ability to secrete other lymphokines (e.g., IL-4, IL-5, γIFN). T cells residing near tissue sites of injury, where platelet activation occurs, are probably being influenced by PDGF.

We believe that a major immunobiologic role played by steroid hormones, specifically those species that undergo end-organ metabolism, is to create and maintain microenvironmental distinctions between discrete lymphoid organs. PDGF, as an example of a locally released bioactive substance, functions to create unique microenvironments anywhere in the body.

**K 014** PATHOLOGICAL EFFECTS OF CYTOKINE EXPRESSION IN VIVO Nora Sarvetnick[1], Judy Shizuru[2], Danling Gu[1], Lise Wogensen[1], Marijo Gallina[1], [1]Department of Neuropharmacology, The Scripps Research Institute, La Jolla, CA 92037, [2]Stanford University, Stanford, CA

Cytokines such as IFN-g are produced during specialized circumstances such as infection. These pleiotropic molecules could potentially be responsible for some of the complex morphological changes associated with infection. We are interested in the pathological consequences of cytokine expression in vivo. We have utilized transgenic mice to target expression of several inflammatory cytokines to the pancreatic beta cells. The expression of IFN-g in the pancreas leads to lymphocytic inflammation and islet cell loss. The islet cell loss is mediated by the lymphocytes that accumulate in the pancreatic parenchyma. Additional morphological changes are observed in the IFN-g expressing pancreas including altered pancreatic vasculature as well as duct cell hyperplasia and islet cell regeneration. By analysis of SCID backcross mice some of these changes were demonstrated to be due to the infiltrating lymphocytes while others are more likely due the direct actions of the cytokine itself. More recently we have been studying the consequences of expression of other inflammatory cytokines on the morphology of the pancreas. In contrast to the studies with IFN-g, expression of the cytokine IL-10 in the pancreas leads to intense inflammation but apparently no significant islet cell loss. The discussed studies are aimed at furthering our understanding of the complex actions of these molecules in vivo.

*Animal Models of Melanoma*

**K 015** IMMUNOBIOLOGY OF PRIMARY MURINE MELANOMAS. Cherrie K. Donawho and Margaret L. Kripke, Department of Immunology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas.

Primary cutaneous melanomas can be induced in inbred mouse strains by applying an initiating dose of a chemical carcinogen (DMBA) to the skin of 4-day old mice, followed by long-term, chronic application of a tumor promoter (Berkelhammer, et al. Cancer Res. 42:3157, 1982). Preliminary experiments suggest that the final incidence of melanomas is strongly influenced by the age at which the initiating dose of carcinogen is applied. Melanomas induced by this method in C3H mice are immunogenic and exhibit a high degree of crossreactivity in *in vivo* immunization and challenge assays. Exposing the mice to UV radiation during carcinogenesis dramatically accelerates the appearance of the melanomas. We are attempting to determine how UV irradiation potentiates melanoma induction by studying the growth of melanoma cells transplanted into UV-irradiated skin. Our recent studies suggest that UV irradiation accelerates the outgrowth of melanoma cells by means of a local, immunosuppressive effect on the skin. Because UV radiation can suppress the induction of contact hypersensitivity (CHS) to contact sensitizing haptens applied to the UV-irradiated site, we investigated whether such an inhibition of the afferent arm of the immune response could be demonstrated at the site of melanoma growth. We found that the protocol of UV irradiation that accelerated the development of melanomas had no effect on the induction of CHS. Furthermore, immunization of mice with melanoma in the UV-irradiated site induced protection against *in vivo* challenge with melanoma cells. Conversely, conditions of UV irradiation that impaired the induction of CHS had no effect on melanoma growth. In contrast, the regimen of UV radiation that enhanced the development of melanomas also inhibited elicitation of the delayed hypersensitivity response to allogenic spleen cells and the rejection of melanomas at the site of UV irradiation in immunized mice. These studies suggest that UV irradiation facilitates melanoma development by interfering with the expression of efferent immunity in the UV-irradiated site. Furthermore, they demonstrate that in addition to its ability to inhibit the induction of CHS, UV radiation can also interfere with the elicitation of certain cell-mediated immune responses, under appropriate conditions.

**K 016** MALIGNANT MELANOMA IN TRANSGENIC MICE, Beatrice Mintz, Lionel Larue, Monika Bradl, Susan Porter, and Andres Klein-Szanto, Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111.

Transgenic mice with the SV40 T-antigen gene driven by the tissue-specific tyrosinase promoter succumbed at a young age to invasive and metastatic melanoma originating in the eyes. At that time, skin melanomas were beginning to appear and were still at an early stage. The properties and longer-term fate of the skin melanocytes have therefore been further analyzed by skin transplantation, by cell culture, and by ultraviolet irradiation in vivo and in vitro. Transgenic melanocytes, which are hypomelanotic in the skin, were explanted from ostensibly normal areas of mice with multiple copies of the transgene and were found to differ from normal in having shorter doubling times, in being able to grow in relatively impoverished media, and in achieving anchorage independence. From the outset, these deviations were more extreme in cell lines with relatively larger numbers of tandem transgene copies. The cells continued to depart further from normalcy during more prolonged culture and were tumorigenic when grafted to immunosuppressed hosts. In contrast, the melanocytes from hemizygous mice with a single copy of the transgene were only slightly different from normal, until a rare spontaneous change leading to tumorigenicity occurred, or until the cells were UV-irradiated. When hemizygous single-copy mice were bred to homozygosity, their explanted cells quickly showed transformed characteristics and were tumorigenic. As these various cell lines of transgenic melanocytes reproducibly undergo a series of changes in culture, leading to malignancy, they provide exceptional material for isolation and identification of genes whose changes in expression are causally related to malignant progression. In addition, the transgene was found in some individuals to express itself in a mosaic fashion in vivo, thereby generating developmental clones of melanocytes with different degrees of propensity for neoplasia. Melanocyte clonal variation in gene expression was also found in other transgenic experiments. Such animals furnish new genetic models for analysis of normal development and of malignancy.

**K 017** THE USE OF CONGENITALLY IMMUNE-DEFICIENT MICE TO STUDY HUMAN TUMOR METASTASES AND IMMUNOTHERAPY, J.J. Mulé, D.L. Jicha, and S.A. Rosenberg, Surgery Branch, NCI, NIH, Bethesda, Maryland 20892.

Malignant melanoma metastasizes to virtually any organ or tissue in patients. In contrast, xenogeneic transplantation of human malignant melanoma subcutaneously or intravenously rarely causes dissemination in athymic (nude) mice, unless metastasizing variants of the original tumor are generated by *in vitro* or *in vivo* selection methods. This lack of metastatic spread has hampered attempts to generate relevant human melanoma models in mice to test the therapeutic efficacy and tumor-homing capacities of immune reagents, such as monoclonal antibodies or autologous human immune effector cells. We have examined the capacity of fresh human melanoma cells to grow, metastasize, and retain phenotypic characteristics in the triple-immune-deficient beige (bg)/nude (nu)/xid (BNX) mouse. This mouse strain is phenotypically and functionally depleted of T cells, B cells, and precursors of LAK cells. Because of the severe immunocompromised status of these animals, it was postulated that human melanoma cells may escape recognition by this mouse strain and thus survive for sufficient time to show organ tropisms similar to those often found in patients. The intravenous injection of fresh single cell suspensions of human malignant melanoma resulted in widely disseminated disease, with numerous tumor nodules identified subcutaneously as well as in a variety of visceral organs including spleen, kidney, thyroid, adrenals, lungs, heart, eye and brain. BNX mouse lymph nodes were consistently replaced by human malignant melanoma. There was no correlation between the percentage of tumor-associated lymphocytes in a given patient's fresh melanoma biopsy and its capacity to metastasize. Widespread organ involvement with injected fresh human malignant melanoma was not observed in athymic (nude) mice. Moreover, in contrast to our experience with human melanoma, single cell suspensions of human colon adenocarcinomas transplanted subcutaneously to BNX mice grew very slowly and failed to disseminate upon intravenous injection. The presence of human melanoma cells in BNX mice was confirmed by FACS analyses using melanoma-specific monoclonal antibodies (mAbs R24 and 48.7) as well as by histology. Metastatic melanoma nodules isolated from a variety of organ sites in BNX mice shared similar FACS staining profiles with the respective patient's original fresh melanoma specimen. Thus, this model should prove relevant in studies to evaluate the therapeutic potency of human melanoma-specific mAbs and immunotoxins as well as new chemotherapeutic agents. We have also found that human melanoma passaged in BNX mice remained lysable *in vitro* by specifically cytolytic human autologous tumor infiltrating lymphocytes (TIL), suggesting that T cell defined tumor antigens were also stably expressed. The adoptive transfer of autologous human melanoma-specific, cytolytic TIL (from two separate patients) in conjunction with the administration of interleukin-2 could significantly improve the survival of mice with disseminated disease when treatment was initiated three days after the intravenous injection of human melanoma cells. Although long-term persistence of transferred TIL was not observed in non-tumor bearing BNX mice, studies are needed to evaluate tumor bearers. Likewise, further investigation is required to determine whether trafficking of human lymphocytes occurs across mouse endothelium in this disseminated melanoma model. Finally, the BNX mouse may prove valuable in studies of the cellular and molecular processes involved in metastatic spread of human melanomas. For example, BNX mice may serve as a useful host to correlate tumor suppressor gene expression (e.g., nm23) in human melanoma with its metastatic potential *in vivo*.

## *Neural Crest Malignancies*

**K 018** MEDULLARY THYROID CARCINOMA (MTC) AS A DISORDER OF NEUROENDOCRINE MATURATION, Stephen B. Baylin, Andrée de Bustros, Douglas Ball, and Barry D. Nelkin, The Johns Hopkins University Medical Institutions, Baltimore, Maryland 21231.

MTC is a prototype neuroendocrine tumor with multiple properties important for the understanding of the biology of the neural crest. This cancer arises from the neural crest C-cells of the thyroid gland, can occur as one of 3 autosomal dominant genetic multiple endocrine neoplasia syndromes, and exhibits maturation defects during tumor progression which involve key molecular steps in neuroendocrine differentiation. The differentiation abnormalities in MTC lead to a decreased capacity for production of the major parent cell polypeptide hormone, calcitonin (CT). The genetic abnormalities involved may include alterations of genes on chromosomes 10 and 1 which are candidates for initiation or progression of MTC respectively. We have developed, as a model for studying neuroendocrine maturation, a culture model for induced differentiation of MTC through insertion of the Harvey ras oncogene. This maneuver activates protein kinase C-mediated signal transduction events, including increased c-jun gene expression, which result in slowing of cell growth, increased transcription of the CT gene with altered splicing of the RNA transcript towards the pattern of normal C-cells, and restoration of mature neurosecretory granules to the cytoplasm. The recognition sites and functions of transcription factors for the CT gene are being identified and include genes which are known to play key roles in developmental events. A tissue-specific enhancer region approximately 1 kb 5' to the transcription start site has been identified which contains 3 essential helix-loop-helix (HLH) consensus sites. Ha-ras gene induced CT transcription results from concerted action of AP-2-like, cyclic AMP response element (CRE)-like and pou-homeo domain consensus sequences at 150 to 200 bp 5' to the start site. The potential role of each of the above molecular steps in the genetics and progression of MTC will be discussed.

**K 019** ONCOLOGIC IMPLICATIONS OF DIFFERENTIAL LINEAGE SPECIFIC GENE EXPRESSION IN EMBRYONAL NEURAL CREST TUMORS, Mark A. Israel,Pediatric Branch,NCI/NIH,Bethesda,MD 20892; Brain Tumor Research Center,University of California, San Francisco, CA 94143-0520.

Some childhood malignancies are thought to arise in embryonic tissues. These tumors present unique opportunities to study the maturation of specific cellular lineages and to examine the possible role of alterations in the regulation of differentiation in tumor development. Several features of neuroblastoma, a tumor thought to arise in cells originating in the embryonic neural crest, suggest that it may be particularly useful in this regard. For example, neuroblastoma has been well-documented to regress and both spontaneous and treatment-induced maturation to a benign tumor, recognizable by the presence of well-differentiated neuronal features, have been described. In studies to examine the role of altered maturation in the development of this tumor, we identified a series of markers that characterize the various cell types of the peripheral nervous system which are recognizable in neuroblastoma tumor tissues. These include cells of the chromaffin, ganglionic, and schwannian lineage. We found that the expression of these markers was temporally regulated and that the patterns of expression that we observed marked recognizable stages in adrenal gland maturation. We then examined the expression of these markers in neuroblastoma tumor cell lines and tissues and found that these could be recognized as corresponding to specific stages of adrenal gland development. During the course of these studies we found that while many neuroblastoma cell lines and tumors seem to grow in response to mitogenic stimulation by IGF-II, the proliferation of only a small subset seems to result from IGF-II mediated autocrine growth stimulation. Interestingly, these malignant tissues express a variety of developmental markers that suggest they arise in cells that express IGF-II physiologically during the course of normal adrenal medullary development.Current experiments directed at understanding the cellular signals by which neural crest cell maturation is mediated may provide insights of therapeutic import, since neuroblastoma tumors corresponding to some stages of differentiation respond very differently to non-specific cytotoxic therapies than tumors corresponding to other stages.

**K 020** CONSTRUCTING MOUSE MODEL FOR NEUROFIBROMATOSIS TYPE I, Tyler Jacks, Earlene Schmitt, and Robert A. Weinberg,Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142

A number of human familial cancer syndromes are caused by the inheritance of a defective copy of a tumor suppressor gene. We are attempting to construct mouse models for these diseases by establishing strains that carry analogous mutations in the murine homologues of these genes. Through the use of gene targeting, we have generated a number of mouse embryonic stem (ES) cell lines with mutations in the *Rb-1*, *p53*, and *NF1* genes. Chimeric animals have been generated through injection of these mutant ES cells into normal host embryos. For *Rb-1* and *NF1* several chimeras have gone on to transmit the ES cell genome to their progeny. These progeny are currently being genotyped to determine which carry the mutant form of *Rb-1* or *NF1*. Heterozygous animals will be analysed for phenotype and crossed to each other to generate nullizygous animals (or embryos).

The *NF1* mutation was designed to best mimic those found in human neurofibromatosis type I patients. The frequently affected fourth exon (according Cawthon *et al.*, *Cell* 62, 1990) has been interrupted by the bacterial neo gene. Targeting was accomplished using the positive-negative selection scheme with a targeting efficiency of approximately one homologous recombinant per 50 G418$^r$ cells. Given this high frequency of recombination, we are attempting to inactivate the second allele of the *NF1* gene by repeating the targeting protocol on heterozygous ES cells using a targeting vector carrying a different selectable gene. These nullizygous cells may prove valuable for biochemical analysis of *NF1* gene function.

*Neurobiology (Joint)*

**K 021** CYTOKINES IN DEMYELINATION AND DYSMYELINATION, Jean E. Merrill[1], Fredricka C. Martin[1], Lisa Thomas[1], Thomas T. Lee[1], Manuel A. Orellana[1], and Irvin S.Y. Chen[2], [1]Department of Neurology, [2]Departments of Medicine and Microbiology and Immunology, U.C.L.A. School of Medicine, Los Angeles, CA 90024.

The demyelinating disease multiple sclerosis (MS) is thought to be the result of autoimmune processes involving overreactive CD4+ T cells which produce Interferon gamma (IFN$\gamma$), Interleukin 2 (IL2), and other cytokines. These T cells activate peripheral blood macrophages which, as a consequence, migrate into the central nervous system (CNS) with the T cells and create inflammation. The macrophages are actively producing Interleukin 1 (IL1) and tumor necrosis factor alpha (TNF$\alpha$). This inflammatory event is associated with demyelination and death of the oligodendrocyte, the glial cell producing myelin. In addition, astrocytes in the lesion or plaque respond by gliosis, an event which includes proliferation and hypertrophy. These glial cells, which do not normally express the class II major histocompatibility antigen (MHCII) or HLA-DR, become DR$^+$ in response to immune inflammation. Microglial cells are bone-marrow derived macrophages; these cells are also activated by this process. Both microglia and astrocytes have been shown to produce IL1 and TNF$\alpha$ in vitro. In vivo in MS lesions, these cells and the invading macrophages also appear to produce these cytokines. Both glial populations respond to IL1 and TNF$\alpha$ by proliferation in vitro. This response, and the in vitro gliosis seen in astrocyte cultures, is the indirect effect of other cytokines induced by IL1 and TNF$_\alpha$. We hypothesize that Interleukin 6 (IL6) and colony stimulating factors are the secondary cytokines induced. IL6 is evident at the lesion edge and blood brain barrier. In addition, IL1 and TNF$\alpha$ are implicated in the destruction of myelin and death of the oligodendrocyte. Transforming growth factor beta (TGF$\beta$) is a natural antagonist of IL1 and TNF$\alpha$. It inhibits microglia cell killing of oligodendrocytes and is seen at the edge of burnt out plaques. If IL1, IL6, and TNF$\alpha$ do play a role in macrophage or glial cell mediated demyelination, TGF$\beta$ might provide an autocrine feedback to downregulate the process. It might explain the limitation of plaque size or possibly be a mechanism for the relapse-remission cycles seen in some MS patients.

CNS AIDS patients experience neurological symptoms which include headache, fever, cognitive changes, abnormal reflexes, and ataxia. More severe disease is characterized by dementia and severe sensory and motor dysfunction. Histological changes include inflammation, astrogliosis, microglial nodule formation, and diffuse dysmyelination in the form of myelin pallor. In 20% of CNS AIDS, frank demyelination is seen. AIDS associated neurological diseases are correlated with greater levels of HIV-1 antigen or genome in brain tissue . In AIDS dementia, macrophages and microglial cells of the CNS are the predominant cell types infected and producing HIV-1. HIV-1 induction of IL1 and TNF$\alpha$, demonstrated in cultures of peripheral blood monocyte/macrophages and glial cells, would lead to an autocrine feedback loop involving further virus replication in T cells and macrophages. IL1, IL6, and TNF$\alpha$ may account for many clinical and histopathological findings in AIDS nervous system disease. Since HIV-1 infected patients produce elevated cytokines in vitro and in vivo in blood, investigations of the CNS for their presence in association with activated macrophages, astrocytes, and microglia is of great importance for a formal association with the clinical and pathological conditions.

**K 022** THE EMERGING NEUROPOIETIC CYTOKINE FAMILY, Paul H. Patterson, Biology Division, California Institute of Technology Pasadena, CA 91125.

As in the hematopoietic system, the enormous variety of phenotypes in the nervous system arises, in part, through the action of instructive differentiation signals. Neuronal culture assays have allowed the identification and cloning of several proteins that control the expression of phenotype-specific genes in developing neurons. The cholinergic differentiation factor (CDF; also known as leukemia inhibitory factor; LIF) induces and suppresses the expression of a number of neurotransmitter synthetic enzymes and neuropeptides in several types of postmitotic neurons. Many of these effects are reversible, and the particular genes affected depend on the type of neuron under study. Ciliary neurotrophic factor (CNTF) alters the expression of the same set of genes in sympathetic neurons as CDF/LIF. The predicted tertiary structures of these two proteins suggest they belong to a family of cytokines that includes IL-6, ONC, MGF and G-CSF. This association is strengthened by the finding that the receptors for CDF/LIF and CNTF are homologous to the transducing and ligand binding subunits, respectively, of the IL-6-R. Moreover, CDF/LIF is a polyfunctional cytokine that shares a remarkable overlap with IL-6 in its actions on non-neural tissues.

To begin to study the role of CDF/LIF *in vivo*, we have localized its mRNA using RT-PCR and RNase protection methods. CDF/LIF mRNA levels are developmentally modulated, and substantial differences are observed between tissues. Particularly high expression is found in the target tissue of cholinergic sympathetic neurons, the footpads that contain sweat glands. Sweat glands were previously shown to induce noradrenergic sympathetic neurons to become cholinergic *in vivo*. Biochemical and immunological analysis indicates that a protein resembling CNTF is also present at the appropriate time in the footpad to mediate the phenotypic conversion that sympathetic neurons undergo during normal development. In addition, CDF/LIF mRNA is selectively expressed in discrete regions of the postnatal rat brain, and in liver and spleen at particular ages. These results support the possibility that CDF/LIF plays a role in hematopoiesis and in the mature CNS.

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*Molecular Genetics of the Neural Crest*

**K 023** MOLECULAR CONTROL OF NEURAL CREST CELL DEVELOPMENT, Jane E. Johnson[1], Susan J. Birren[1], Kathryn Zimmerman[1], Tetsuichiro Saito[1,2], Li Ching Lo[1,2], Joseph Verdi[1,2] and David J. Anderson[1,2], [1]Division of Biology 216-76, [2]Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125.

Neural crest cells migrate from the apex of the neural tube to a variety of locations in the embryo before generating a diverse array of differentiated derivatives. The molecular mechanisms underlying neural crest cell lineage commitment and differentiation are for the most part unknown. Current evidence is consistent with the idea that the development of the neural crest involves mechanisms similar to those that operate during hematopoiesis. The earliest crest cells are multipotent, but become progressively restricted in their developmental potential. In some sublineages of the neural crest, committed progenitor cells have been identified. These cells have a restricted repertoire of developmental capacities and choose their fates according to signals in their local environment. One such progenitor that has been isolated and studied in detail is the sympathoadrenal (SA) progenitor. This cell migrates to the adrenal gland and develops into a chromaffin cell under the influence of adrenal corticosteroids, or aggregates to form ganglia in the sympathetic chain where it develops into a sympathetic neuron. Immortalized cell lines have been established from this progenitor by retroviral transduction of the v-*myc* oncogene. These lines, called MAH cells, appear to have retained many of the properties of their normal counterparts. They have been used to study the mechanisms whereby developing sympathetic neuroblasts become first responsive to, and then dependent upon, nerve growth factor (NGF), the prototypic neurotrophic factor. MAH cells have also been used to examine additional developmental potentials that may be available to SA progenitors. These studies, taken together with other recent data, support the idea that the SA progenitor may be a multipotent "master" neuroendocrine progenitor for the peripheral nervous system. Finally, candidate determination genes for the SA lineage have been isolated from MAH cells. One such gene is MASH1, a vertebrate homologue of the *Drosophila* neurogenic determination genes *achaete-scute*, which are members of the helix-loop-helix family of transcriptional regulatory genes. Recent evidence indicates that MASH1 is a transcription factor that is transiently expressed by restricted subpopulations of neural precursor cells, consistent with the idea that it may be a vertebrate neuronal determination gene.

**K 024** *Abstract Withdrawn*

**K 025** PROTOONCOGENES IN THE NEURAL CREST: NEUROTROPHIN RECEPTORS, Dan Soppet[1], Pantelis Tsoulfas[1], Dave Kaplan[2], Enrique Escandon[3], Barbara Hempstead[4], Arnon Rosenthal[3], Karoly Nikolics[3], Moses Chao[4], and Luis F. Parada[1], [1]Molecular Embryology Section, and [2]Eukaryotic Signal Transduction, ABL-Basic Research Program, Frederick, MD, 21072, [3]Genentech, South San Francisco, CA, [4]Cornell Medical College, New York, NY.

We are studying a family of closely related tyrosine kinase (TK) receptors. To date we have identified three *trk* TK receptors (*trk*, *trkb*, *trkc*). All receptors display expression predominantly in cells of the central and peripheral nervous systems. During embryonic development, *trk* is primarily found in neural crest-derived sensory neurons of the PNS (trigeminal, superior, jugular, and dorsal root ganglia). *trkb* and *trkc* are more related by sequence to each other than either is to *trk*. Moreover *trkb* and *trkc* exhibit more widespread expression patterns and encode for at least two distinct gene products: a full length TK receptor and a truncated version lacking the TK catalytic domain. We have recently demonstrated that the *trk* gene products encode the receptors for the neurotrophic factors: NGF (nerve growth factor); BDNF (brain derived neurotrophic factor); NT-3, and NT-5 (neurotrophins 3 and 5). The relationships between receptors and ligands are complex but provide novel and unexpected insights into the biology and signal transduction of these factors.

**K 026** TUMOR SUPPRESSOR GENES AND NEURAL CREST-DERIVED NEOPLASMS: TOWARD THE ISOLATION OF THE GENE CAUSING NEUROFIBROMATOSIS TYPE 2 (NF2), Bernd R. Seizinger[1], Nikolai Kley[1], Anil Menon[1], Terry Lerner[2], Mia MacCollin[2], James Troffater[2], Jonathan Haines[2], James Gusella[2], Ronald Lekanne-Deprez[3], Ellen Zwarthoff[3], [1]Molecular Neuro-Oncology Laboratory, [2]Molecular Neurogenetics Laboratory, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, U.S.A., [3]Department of Pathology, Erasmus University, Rotterdam, The Netherlands.

Many human cancers are known to occur in two different forms: as sporadic tumors in the general population, and as hereditary tumors within families. Hereditary tumor syndromes offer unique model systems for isolating genes whose mutations lead to cancer. There is accumulating evidence that hereditary and sporadic tumors are caused by similar pathogenetic mechanisms affecting the same genes. The cloning and characterization of these genes may therefore have important implications for diagnosis and treatment not only of the relatively rare hereditary tumors, but also for their much more common sporadic counterparts.

Recombinant DNA technology has provided a powerful strategy for investigating inherited diseases in which the defective protein is unknown. This strategy has been termed "reversed genetics" because the first goal is not to find the defective protein, but rather to isolate the defective gene based on the determination of its chromosomal location in the human genome. However, most genes which have been cloned thus far using the "reversed genetics" approach, have been isolated with the additional aid of cytogenetically visible chromosomal aberrations, providing potential short-cuts to the defective genes. For example, the gene for neurofibromatosis type 1 (NF1) was recently identified by cloning the translocation breakpoints of two NF1 patients with constitutional translocations in the NF1 region on chromosome 17q11.2 (see first speaker).

NF is one of the most common inherited disorders affecting the human nervous system. There are two clinically and genetically distinct forms of NF: NF1 (von Recklinghausen NF) and a less frequent form, NF2 (bilateral acoustic NF). Although different cell types may be affected, the most common abnormalities in both NF forms are in the nervous system, in cells of neural crest origin. Acoustic neuromas, Schwann cell-derived tumors of the 8th cranial nerve, are the hallmark of NF2 and frequently lead to deafness. Furthermore, meningiomas and other neural-crest derived tumors such as spinal Schwannomas, are common in NF2 patients. We have previously shown that the gene causing NF2 maps to chromosome 22q. The region containing the NF2 gene is frequently deleted in tumors associated with NF2, in both their sporadic and hereditary forms, including acoustic neuromas, spinal Schwannomas, and meningiomas, suggesting that the NF2 gene belongs to the family of "tumor suppressor" genes, i.e., genes which normally confer growth suppression, and whose loss of function is associated with tumor formation.

The recent discovery of a meningioma tumor specimen with a translocation on chromosome 22 (Zwarthoff et al.) has provided a potential short-cut to the isolation of the NF2 gene. We have shown that the translocation breakpoint maps to the region on chromosome 22 identified as the NF2 region, based on tumor deletion and linkage studies. We have meanwhile identified flanking markers of the translocation breakpoint which recognize the translocation breakpoint on pulsed-field gel electrophoresis. These and other markers also appear to identify constitutional aberrations in patients with NF2 and multiple meningiomas. Thus, the search for transcripts in this region which are expressed in normal Schwann cells, but not in Schwann cell-derived tumors, may eventually lead to the identification of the NF2 gene and/or the or a meningioma "tumor suppressor" locus on chromosome 22.

## *MHC Molecules and Their Biologic Role*

**K 027** GENES AND TRANSCRIPTION FACTORS THAT ARE REGULATED BY N-MYC IN NEUROBLASTOMA, Rene Bernards, Anil K Rustgi, Laura van 't Veer, Guus Hateboer, Roderik Beijersbergen, Cheryl Rich and Marc Billaud. Division of Molecular Genetics, The Cancer Center of the Massachusetts General Hospital, Charlestown MA 02129

In neuroblastoma, amplification of the N-myc oncogene is closely correlated with increased metastatic ability. The N-myc protein shares significant homology with a number of transcription factors that are implicated in the control of cell proliferation and differentiation. It is thus likely that N-myc contributes to neuroblastoma tumor progression by altering the expression of a number of key cellular genes, the altered expression of which is ultimately responsible for the N-myc-induced changes in cellular behavior.

We have studied the effects of N-myc expression on neuroblastoma phenotype by transfecting a rat neuroblastoma cell line with the N-myc oncogene. We found that N-myc elicits a number of responses in these neuroblastoma cells that are reminiscent of advanced stage human neuroblastoma such as increased growth rate and increased metastatic ability.

We have studied the effect of N-myc on neuroblastoma gene expression and found that N-myc suppresses the expression of MHC class I antigens, neural cell adhesion molecule (NCAM) and the δ isoform of protein kinase C. In each case, suppression of gene expression required the continued expression of high levels of N-myc, suggesting that N-myc was responsible for the observed changes in cellular gene expression.

The N-myc mediated suppression of MHC class I gene expression appears to be caused by a reduction in the transcription of MHC class I genes in N-myc-amplified neuroblastoma. Transfection of murine MHC class I gene promoter constructs into neuroblastoma cells, in combination with electrophoresis mobility shift assays (EMSA), revealed that N-myc suppresses the expression of MHC class I genes by reducing the binding of a nuclear factor, named H2TF1, to an enhancer element in the MHC class I gene promoter. As a result of this, the MHC class I gene enhancer is virtually completely inactivated in N-myc-amplified neuroblastomas. Our recent studies suggest that the H2TF1 transcription factor consists of a homodimer of the p50 subunit of the NF-κB transcription factor. Our data further indicate that the gene encoding the p50 protein, which is related to the c-rel proto oncogene encoded protein, is suppressed in its expression by N-myc. Recent work from others indicates that N-myc functions by forming a heterodimer with a second protein, named MAX, which allows N-myc to bind to DNA in a sequence-specific fashion. We have constructed a mutant MAX protein, named ΔMAX, which has retained the ability to form a heteromdimer with N-myc, but has lost the ability to bind to DNA. We would therefore expect that the ΔMAX protein could interfere with N-myc protein function by inhibiting its DNA binding. The biological effects of over-expression of the ΔMAX protein in N-myc-amplified neuroblastomas, including its effect on tumorigenicity and metastatic ability, will be presented.

Myc proteins appear to be modular transcription factors: the caboxyl terminus of myc proteins is required for dimerization and DNA binding, whereas the amino terminus mediates the trans-activating function of myc proteins. We have recently shown that amino terminal domains of both c-myc and N-myc proteins can mediate binding to the product of the retinoblastoma gene, pRB. Data will be presented that indicate that the mammalian TATA binding protein, TFIID, also interacts with amino terminal domains of myc proteins. A model will be presented in which pRB controls trans-activation by myc proteins.

**K 028** TUMOR PROGRESSION AND STEALTH IN MELANOMA: THE ROLE OF HLA-CLASS II MOLECULES, DuPont Guerry, Michael A. Alexander, Jeanette L. Bennicelli, and William M.F. Lee, Cancer Center, Pigmented Lesion Group, and Hematology-Oncology Section, Dept. of Medicine, 7 Silverstein Bldg., Hospital of the University of Pennsylvania, 3400 Spruce St., Philadelphia, PA 19104.

Melanoma evolves in stepwise fashion. Evident lesional steps in tumor progression include common acquired nevi, dysplastic nevi, the radial and vertical growth phases of primary melanoma, and metastatic disease. A variety of observations suggests that biologically early melanoma evokes an antigen-driven, T cell response which becomes attenuated with the evolution of the metastatic phenotype. We have suggested that melanoma cells of early disease (dysplastic nevi, radial growth phase melanoma, and some vertical growth phase lesions) act as competent antigen presenting cells, directly presenting tumor-associated or model (tetanus toxoid, TT) antigens in association with HLA-DR class II molecules to CD4+ T cells. This function is lost with tumor progression. We hypothesized that this loss was due to acquired dysfunction of class II molecules and demonstrated in antigen presentation studies with affinity purified HLA-DR molecules in an artificial membrane system that these molecules isolated from metastatic (but not primary) melanoma cells fail to present antigen (TT). To further implicate class II dysfunction in metastatic melanoma we have transduced tumor cell lines with a retroviral vector containing full-length HLA-DRB cDNAs. Such melanoma cells have been examined by Northern analysis and found to express both endogenous and transduced HLA-DRB transcripts. Cell surface expression of HLA-DR molecules was demonstrated by flow cytometry using a DR type-specific monoclonal antibody. Class II gene transfer and expression resulted in restoration of function in two assays. First, a DR expressing metastatic melanoma cell, previously unable to present antigen and transduced with a DR-matched B gene, successfully presented TT to an antigen-specific, DR-matched, T cell clone. Second, another comparably treated metastatic cell line was newly able to stimulate the proliferation of autologous, peripheral blood T cells, absent the addition of any exogenous antigen. These findings suggest that in the evolution of the advanced disease phenotype "natural selection" favors variant tumor cells with defective capacity for antigen presentation. Such cells do not present tumor-associated antigens to T cells in the afferent arm of a cellular immune response. In other systems pre-clinical studies have demonstrated: 1) protection against challenge with class II negative tumor cells by administration of syngeneic tumor cells genetically engineered to express class II antigens; and 2) rejection of established tumors triggered by antigen-specific, cloned, helper T cells. Taken together, these observations suggest a new therapeutic strategy: the expression of functional class II molecules by a subpopulation of tumor cells reintroduced to the host. This manipulation should allow inspection by helper T cells of the tumor cell surface for immunogenic antigens and the subsequent, multi-pathway, immunological rejection of both engineered tumor cells and their stealthy co-conspirators.

## *Immunotherapy of Melanoma and Other Neural Crest Tumors*

**K 029** MONOCLONAL ANTIBODY THERAPY OF NEUROBLASTOMA AND MELANOMA. Nai-Kong V. Cheung, Memorial Sloan-Kettering Cancer Center, New York.

Monoclonal antibody (MAb) can potentially target complement, leukocytes, radioisotopes, toxins and other cytotoxic agents to melanoma and neuroblastoma cells. The clinical use of MAbs has, in general, exploited antigens of restricted distribution, with high tumor expression and relative homogeneity. These tumor cells are sensitive to antibody mediated lysis (ADCC, antibody dependent cellular cytotoxicity and CMC, complement dependent cytotoxicity) and are often vulnerably positioned (metastatic disease in blood, marrow and lymph nodes) for optimal antibody binding as well as requisite contact with complement proteins and leukocytes. Phase I studies of mouse MAbs have demonstrated their ability to reach tumor sites after systemic administration. Toxicities have been mild and maximal tolerated doses are still being defined. ADCC and CMC are potential anti-tumor mechanisms in vivo, since 1) antibodies of IgG subclasses that carry no effector functions have failed to effect clinical responses and 2) significant anti-tumor effects are elicited only in rodents whose complement and leukocytes can utilize these MAb for tumor cytoxicity. While HAMA is detectable in most patients undergoing Mab treatment, patients with prior intensive chemotherapy (as in most patients with neuroblastomas) can be repetitively challenged without mounting significant HAMA titers. Xenogenization of tumor cells by MAb or the induction of anti-idiotypic antibodies may potentially lead to active tumor immunity. The use of anti-idiotypic MAb to induce active host anti-tumor response is being tested in clinical trials. An increasing number of cytokines have been shown to stimulate the number and activity of effector cells. A rational combination of these cytokines and MAbs may be potentially useful in the treatment of these chemo-resistant tumors.

**K 030** THE ROLE OF INTERFERONS IN THE THERAPY OF MELANOMA, John M. Kirkwood, Department of Medicine, Division of Medical Oncology, University of Pittsburgh Medical Center and Pittsburgh Cancer Institute, Pittsburgh, PA

Interferons (IFNs) have been the most broadly applied biological agent in the past decade for the investigational therapy of human melanoma. Antitumor activity has been observed for nonrecombinant and recombinant IFNs, including IFN alpha (α), beta (β) and gamma (γ). Direct anti-proliferative effects of IFNs are well documented in vitro, but efforts to predict clinical responsiveness by in vitro assays against melanoma have not been rewarding thus far. In addition, immunomodulatory activity of IFN has been well documented, but no correlation between this indirect activity and clinical antitumor activity has been established.

The largest clinical experience in melanoma to date has been gained with IFN alfa-2, achieving objective responses in ≈20% of patients, up to a third of which are complete and durable. This clinical evidence of antitumor activity in metastatic melanoma has led to the initiation of 2 large multicenter studies of IFN alfa-2 in 1984. These trials utilized maximally tolerable dosages of IFN alfa-2 administered for 3 months (North Central Cancer Treatment Group) and 1 year (Eastern Cooperative Oncology Group, ECOG). Both trials completed accrual in the past year with 273 and 285 subjects, respectively. Interim analyses of the latter study were sufficiently encouraging that a follow-up trial has been initiated, further testing the efficacy of high-dose IFN alfa-2 for 1 year and examining the potential role of lower immunomodulatory dosages of IFN alfa-2 for >2 years, as compared to a control arm of observation. Recent studies in the WHO Melanoma Program are also testing the role of low doses of IFN alfa-2 for 2 years compared to control. These trials, which are being performed in conjunction with laboratory studies of the in vitro anti-proliferative, antigen-modulating, and immunomodulating function of IFN alfa-2, may provide answers to the question of the mechanism(s) of IFN alfa-2 action against melanoma, as well as its clinical role as an adjuvant therapy for melanoma.

IFNγ has also been examined considerably as an immunomodulator for the therapy of melanoma. However, its clinical activity as an antitumor agent appears to be somewhat less than IFN alfa-2 in metastatic disease. One adjuvant trial with IFNγ has been concluded in the Southwest Oncology Group with negative results. Extensive dose-response studies in our group and in the ECOG at large indicate that the immunomodulatory functions of IFNγ appear to differ at high and low dosage, affecting NK and monocyte functions at high dosage and T cell distribution and functions at low dosage.

A preclinical basis for combined usage of IFNs with antibodies, cytotoxic drugs, and IL-2 lies in their in vitro synergy. Early clinical trials of IFN alfa-2 with monoclonal antibodies, cytotoxic chemotherapy, and IL-2 have all yielded encouraging preliminary data. We have recently completed a phase I trial of IFNγ combined in sequence with IFN alfa-2, in which improved antitumor effects were associated with immunological effects upon the T cell compartment and reduction of the CD8+ circulating T cell subset. This trial, and similar evidence of altered numbers and/or functions of the T cell, suggests that the IFNs, like the interleukins, may achieve their effects upon melanoma and other tumors through specific host immune response mechanisms.

**K 031** USE OF T CELL GROWTH FACTORS IN THE TRATMENT OF MELANOMA, Michael T. Lotze, Nelly E. Villacreses, Joshua T. Rubin, M.D., Howard D. Edington, Walter J. Storkus, Elaine M. Elder, Herbert J. Zeh, Barbara A. Pippin, Theresa L. Whiteside, Eckhart Weidman, and Ronald B. Herberman. University of Pittsburgh, Pittsburgh, PA.

Melanoma represents the single best example of a human tumor for which specific T-cell reactivity can be identified. Perhaps one of the most compelling pieces of evidence demonstrating their specific role is the responsiveness of patients with metastatic melanoma to treatment with the prototypic T-cell growth factor, Interleukin-2 (IL-2). Approximately 25% of patients will manifest a partial or complete response to high- dose IL-2 therapy, limited by the development of a capillary leak syndrome. Histologic examination of responding tumors demonstrates profound infiltration by approximately equal numbers of CD4 and CD8 positive T-cells to a degree far greater than observed prior to therapy. The precise role of T-cells in the evolution of the neoplasm from the earliest precursor nevi remains to be defined. Although combination treatment of IL-2 with alpha-interferon have been associated with apparent enhanced response rate, this is with a substantial increase in associated cardiac and neurotoxicities. Although no clear-cut relationship between responsiveness to IL-2 based therapy and inherited expression of individual HLA Class I and Class II alleles has been demonstrated, there appears to be a modest increase in the frequency of HLA-DQ1 (94% of 31 responders tested vs. 54% of the general population), and of HLA-A19 (37% of 35 responders vs. 27% of the general population). Major efforts are currently underway to increase the response rates to IL-2 by the addition of additional cytokines or the adoptive transfer of tumor infiltrating lymphocytes. To date, no specific therapy has been shown to be substantively better. IL-4 administration, in our trials conducted in the Surgery Branch of the National Cancer Institute, was not associated with anti-tumor responses. Combinations trials with Interleukin-2 indicated that although substantive anti-tumor effects could be observed, that no apparent major increase in responsiveness of melanoma or other tumors was observed. Since enhanced expansion of tumor infiltrating lymphocytes is mediated by growth in combinations of low- dose IL-2 and IL-4, we have proposed a study evaluating the trafficking of such expanded lymphocytes following adoptive transfer in association with Interleukin-2 and Interleukin-4. We have been able to establish several TIL cultures from fresh tumor samples, to maintain them in long-term culture, and to gene mark them using the LNL6 retroviral vector with successful incorporation of the neomycin phosphotransferase gene. Such TIL's appear to demonstrate no notable alterations in phenotype or cytolytic activity when compared to their non-transduced counterpart. These studies have been approved by the Recombinant DNA Advisory Committee, and will be initiated soon at the University of Pittsburgh Medical Center. Selection requires a slow increase in the concentration of the neomycin analogue, G418. Although we are in the process of identifying the individual V-alpha and V-beta chains used in oligoclonal populations of expanded T-cells, as well as the fresh tumors from which they are derived, as of yet, no convincing evidence of increased expression of any of the individual examined variable regions have been noted. There are a variety of other novel cell growth factors which are now available for evaluation in pre-clinical and clinical trials. IL-7 induces human lymphokine-activated killer cell activity from human peripheral blood mononuclear cells, and when administered to mice, increases primarily proliferation of B-cells, but as well an expansion of T-cells in the spleen and lymph nodes. Pre-clinical studies in murine models have failed to demonstrate significant anti-tumor effects of IL-7 alone, although attempts to expand TIL's, both in man and murine models, are underway. IL-10 was originally defined as a factor inhibiting Interleukin-2 and gamma-Interferon production by T-helper 1 cells (cytokine synthesis inhibitory factor). A truncated version of IL-10 found within the EBV genome, BCRF1, has this activity, but unlike murine IL-10, has no TCGF activity. Although IL-10 has been demonstrated to markedly synergize with IL-2, IL-4, and IL-7 in the growth of thymic and peripheral T-cells in the mouse, no such activity has yet been demonstrated in human cells. Interleukin-12 is a heterodimeric protein which enhances proliferation of T-cells and large granular lymphocytes, synergizing with IL-2 in inducing cytolytic activity. It markedly enhances the production of gamma-Interferon, and appears to serve as a B-cell-derived factor capable of partially supporting T-cell growth. Studies of IL-12 in expanding T-cells from human melanoma are underway.

Evidence supporting a unique or limited number of melanoma antigens shared by tumors derived from a number of individuals is suggested by evidence from many laboratories of lysis by T-cells clones in lines of tumors bearing a common restricting MHC antigen. In our studies melanomas appear capable of processing and presenting influenza antigen and/or peptide to flu-specific T-cells. Strategies to identify the putative melanoma antigen(s) using transfection with mutant HLA-A2 genes, as well as peptide stripping experiments, are underway. The future development of cytokine base therapies will be dictated by not only the availability of specific antigens identified in melanoma, but also the ability to direct T-cells to sites of tumor using immunization with either peptides or tumor transfected with the cytokine genes including IL-2 or other T-cell growth factors.

*Melanobiology*

**K 100** MELANOTRANSFERRIN AND HUMAN MELANOCYTE DIFFERENTIATION, PROLIFERATION AND TRANSFORMATION. Jerry Bash, Department of Medical Laboratory Sciences, Florida International University, Miami, FL 33199.
Normal human epidermal melanocytes (NHEM) cultured in the presence of phorbol myristate acetate (PMA) express the melanoma associated antigen melanotransferrin (p97) in conjunction with rapid proliferation and bipolar dendritic morphogenesis. Melanoma cells, by contrast, constitutively express p97 in high density without requirement for PMA stimulation and rarely exhibit dendritic morphology. In the present study, the relationship between p97 expression and proliferation of melanocytes and melanoma cells was further examined to test the hypothesis that melanotransferrin plays an active role in both melanocyte differentiation and transformation. NHEM (Clonetics, Inc., San Diego, CA) were cultured in melanocyte basal medium (MBM, Clonetics) supplemented with basic fibroblast growth factor, bovine pituitary extract, bovine insulin, hydrocortisone and varying amounts of PMA. NHEM growth (cell counts and $^{3}$H-thymidine incorporation and dendrite extension (phase contrast photomicroscopy) were observed to correlate with p97 expression (immunoperoxidase assay). The human melanoma cell line SK-MEL-28 (ATCC, Rockville, MD) which expresses a high density of p97 was studied in parallel. NHEM cultured in the absence of PMA or in supraoptimal PMA showed little proliferative capacity, dendrite extension or p97 expression, but could be induced to express these properties in the presence of spent medium from SK-MEL-28 cultures. Similar results were obtained by the addition of monoclonal anti-p97 (ME 9-61, Wistar). These effects were not synergistic but could be enhanced by addition of supoptimal PMA. These observations are compatible with the hypothesis that p97 may normally function as a receptor for a transiently produced growth factor. Unregulated production of the factor during melanocyte transformation, in conjunction with p97 upregulation may achieve an autocrine feedback loop contributing to malignant transformation.

This work was partially supported by a Summer Research Fellowship from Florida International University, Miami,FL

**K 101** HUMAN MELANOMA EXPRESS AND SECRETE A DEVELOPMENTALLY REGULATED NEUROTROPHIC GROWTH FACTOR, Nicola Hartmann, WJ. Fang, M. Herlyn*, U. Rodeck*, M. Bano, J. Sasse[§] and A. Wellstein, *Wistar Institute, Philadelphia, PA; [§]Shriner's Hospital, Tampa, FL and Lombardi Cancer Center, Georgetown Univ., Washington DC.

Growth factors with paracrine activity play an important role during tumor growth. We studied human metastatic melanoma cell lines and detected activity by soft agar colony formation of an epithelial (SW-13) and a fibroblast cell line and by monolayer growth of endothelial cells. An activity profile similar to one derived from human breast cancer cells (MDA-MB 231) was identified as heparin-binding growth-associated molecule / pleiotrophin (HB-GAM/PTN) (1), a developmentally regulated neurotrophic growth factor (2,3). Partial purification of the activity from melanoma cells (WM239A) was achieved by heparin affinity chromatography. Western blotting of the supernatant from these cells with a rabbit antiserum raised against the N-terminal sequence of HB-GAM/PTN showed an 18 and a 14 kDa species. This finding is in agreement with C-terminal cleavage of HB-GAM/PTN, isolated from bovine brain (4). Experiments with cell extracts showed corresponding bioactivity in the endothelial cell growth assay and immunoreactivity in an ELISA after heparin-affinity chromatography. PCR amplification confirmed the expression of mRNA for HB-GAM/PTN in the WM239A cell line. Furthermore, a 1.4 kb mRNA species was easily detected in a Northern blot indicating a high level of gene transcription. Preliminary data show that another human metastatic melanoma cell line (WM852) also produces this growth factor and melanocytes do not. We speculate that this novel growth factor is transformation-associated and may function as a tumor angiogenesis factor due to its endothelial cell stimulating properties.

(1) Wellstein et al., JBC, in press; (2) Merenmies and Rauvala, JBC, 1990, 265:16721; (3) Li et al., Science, 1990, 250:1690; (4) Boehlen et al., Growth Factors, 1991, 4:97. Supported by the Deutsche Forschungsgemeinschaft

**K 102** CHARACTERIZATION OF CELLULAR RESPONSES TO A RADIATION RESISTANCE FACTOR ELABORATED BY MELANOMA CELLS. Helene Z. Hill, George J. Hill, Muhammed Ali and Uwe Schlehaider, Departments of Radiology and Surgery, New Jersey Medical School, Newark, NJ 07103-2714
Melanomas are generally considered to be very resistant to low LET radiation. Our studies of clonally related cell lines of Cloudman S91 mouse melanomas have demonstrated that in tissue culture these cells elaborate a diffusible factor which decreases the radiation sensitivity of a radiation sensitive target cell line (S91/amel). The cell line that is most proficient at elaborating the factor, S91/I3, is also the most radioresistant (H.Z. Hill, Z. Trizna, M. Ali and G.J. Hill, **Radiation Research**, in press). We have now further characterized cellular responses to the factor. The target cells remain responsive to the factor for as long as 4 days after irradiation. In split dose studies, if no factor is present, there is a slight but gradual increase in survival with increase in time between the doses. If the factor is present, there appears to be a rapid increase in recovery that is maximal at between 2 and 4 hours. This rapid increase occurs even if the resistance factor is added to the medium one day after the cells have been irradiated. These studies reveal that there are 2 aspects to split dose recovery. The first - which occurs within a short period of time after irradiation - probably involves repair of genomic damage. The second - controlled by the resistance factor - involves some additional, yet to be defined, aspect of cellular recovery. Potentially lethal damage in these cell lines was studied by exposing cells to radiation in high salt buffer for 20 minutes. The radiation resistance factor did not influence the extent of recovery, indicating that it does not operate at the level of the sort of genomic repair revealed by this type of study. The target S91/amel cells are also more resistant to UVC irradiation when plated in the presence of the factor after irradiation. Our studies suggest that there are cellular factors that operate in recovery from radiation damage beyond the repair of DNA and that such a factor or factors may be important in the refractory responses of melanoma cells to various therapies.

**K 103** THE IDENTIFICATION AND CHARACTERIZATION OF PROGRESSION ASSOCIATED MOLECULES IN HUMAN MELANOMA. Judith P. Johnson, Ute Rothbächer, Simone Stratil, and Gert Riethmüller, Institute for Immunology, University of Munich, Goethestrasse 31, 8000 Munich 2, Germany.
The development of malignant melanoma from epidermal melanocytes and its progression to metastatic disease is thought to occur in a step-wise process which is reflected in the existence of a series of morphologically and clinically distinct melanocytic lesions. In order to identify the changes in gene expression characterizing this process, we have produced monoclonal antibodies (MAbs) which demonstrate differential reactivity with these various lesions. Using this approach, several molecules which are expressed by metastatic lesions but which are only rarely detectable on benign melanocytes have been identified. Some of these molecules, such as the cytoplasmic p76 (MUC54) and the surface glycoprotein CLIP.M, are early progression markers and first become detectable on histologically abnormal nevi. They are strongly expressed on all stages of malignant tumors and may define molecules which contribute to transformation of melanocytes. Others, such as the molecules identified by MAbs 6G6, 3A11, 1H11, MUC18 and P3.58, are detectable only on advanced primary tumors and metastases. These so called late progression markers may represent molecules which contribute to the development of metastatic potential in the tumor. Interesting, cDNA cloning of 2 of these markers (MUC18, P3.58) revealed them both to be cell adhesion molecules. P3.58 was found to be identical to the leukocyte adhesion molecule ICAM-1. MUC18 expression in normal tissue is limited to some smooth muscle cells. The molecule is unique and has the greatest seqeunce similarity to DCC (deleted in colon carcinoma), carcinoembryonic antigen, and NCAM, all of which have been shown to mediate intercellular adhesion.

**K 104** HISTOPATHOLOGICAL STUDY OF UVEAL MELANOMAS AND ITS METASTASES

G.P.M. Luyten*, J. Post**, C.M. Mooij***, T.M. Vroom****, P.T.V.M. de Jong**

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Malignant tumors are known to be composed of heterogenous cell populations. During tumor progression more invasive and aggresive subpopulations arise with metastatic potential. Celltype is one of the major prognostic factors in uvea melanoma. Occurrence of epitheloid cells in the primary tumor is associated with a higher tumor-related mortality. However metastatic disease can occur in patients with a pure spindle cell melanoma. Therefore we have retrospectively studied a group of patients with uvea melanoma who were treated with enucleation and who died of metastatic disease. In this group we compared the histopathological celltype of the primary tumor and its metastases.

**K 105** STEEL FACTOR IS REQUIRED FOR MAINTAINENCE, BUT NOT DIFFERENTIATION, OF MELANOCYTE PRECURSORS IN THE MOUSE NEURAL CREST, Mark Murphy, Kate Reid, *Douglas E. Williams, *Stewart D. Lyman and Perry F. Bartlett, Walter and Eliza Institute of Medical Research, Royal Melbourne Hospital, Parkville, 3050, Australia and *Immunex Research and Development Corporation, Seattle, Washington 98101.

Skin melanocytes are derived from neural crest cells that migrate into the dermis during embryogenesis. Two mouse mutants, *Steel* and *White dominant-spotting*, which have defects somewhere in the pathway of melanocyte production, have recently been shown to have deletions in the genes that code for a new growth factor, Steel factor (SLF), and its receptor, respectively. Here, we have investigated the role that SLF plays in melanogenesis using cultures of mouse neural crest and found that its primary action is the maintainence and/or proliferation of melanocyte precursors. It has no effect on the final stage of melanocyte differentiation, the production of melanin, which appears to require a further factor whose action is mimicked by the phorbol ester, TPA.

**K 106** $pp60^{c\text{-}src}$ IN HUMAN MELANOCYTES AND MELANOMA CELLS EXHIBITS ELEVATED SPECIFIC ACTIVITY AND MODIFIED PHOSPHORYLATION COMPARED TO HUMAN FIBROBLAST $pp60^{c\text{-}src}$, Tracy J. O'Connor[1], Eleanor Neufeld[1], John Bechberger[2], and Donald J. Fujita[1,2], Cell Regulation Group, Department of Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada T2N 4N1[1], Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5B7[2]

Experiments in our laboratory suggested that there was an elevated level of $pp60^{c\text{-}src}$ tyrosine kinase activity in certain human melanoma cell lines compared to human foreskin fibroblasts. In order to determine whether this was a tumor-specific or a tissue-specific phenomenon, we looked at $pp60^{c\text{-}src}$ activity in normal human epidermal melanocytes. It was found that they contained $pp60^{c\text{-}src}$ with a seven-fold elevation in specific activity compared to human foreskin fibroblasts. $pp60^{c\text{-}src}$ from melanocytes was the same size as the fibroblast form; therefore it was not neuronal $pp60^{c\text{-}src+}$. Melanocyte $pp60^{c\text{-}src}$ had a reduced level of carboxy-terminal tyrosine phosphorylation, which might contribute to its elevated specific activity. These results suggest that activation of $pp60^{c\text{-}src}$ may be involved in normal melanocyte growth, differentiation, or function.

**K 107** NEURAL CREST DIFFERENTIATION OF HUMAN PROSTATE CARCINOMA CELL LINES: EVIDENCE THAT PROSTATE CARCINOMA CELLS ARE CAPABLE OF EPITHELIAL AND NEURAL CREST DIFFERENTIATION, Jane B. Trepel, Farzaneh Pirnia, Oliver Sartor, Luke Whitesell, Won-Ki Kang, Michelle Sheahan, Charles E. Myers and Yung-Jue Bang, Clinical Pharmacology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

While studying the signal transductions that regulate the growth of human prostate carcinoma cells, we found that elevation of intracellular cyclic AMP (cAMP) through addition of cAMP analogs or phosphodiesterase inhibitors caused growth arrest in the four cell lines studied, and in two cell lines caused a striking shift from an epithelial to a neuronal morphology. Phenotypic examination of the four prostate adenocarcinoma cell lines showed that prior to elevation of cAMP each cell line expressed at least two markers associated with neural crest differentiation, including neuron specific enolase, as detected by cytochemical and Western blot analysis; S100, determined by cytochemical analysis; and low and intermediate molecular weight neurofilament proteins, detected by Western blot analysis. Markers of neural crest differentiation were stable or increased following treatment with cAMP-elevating agents, while the epithelial marker HEA 125, and stage specific embryonic antigen 4 expression were down-regulated. Transmission electron microscopy demonstrated that in the androgen-sensitive prostate carcinoma cell line LNCaP, cAMP-induced neuronal morphology was accompanied by the appearance of dense-core granules. High levels of expression of the nonreceptor protein-tyrosine kinase $pp60^{c\text{-}src}$ have been associated with neural crest differentiation. All four cell lines expressed readily detectable $pp60^{c\text{-}src}$ as determined by Western blot and/or immune complex kinase assay. Addition of dibutyryl cAMP or the phosphodiesterase inhibitor IBMX caused an increase in *src* activity. This increase appeared to be associated with neural crest differentiation rather than growth inhibition, because addition of TGF-β1, which was highly growth inhibitory but did not induce neuronal morphology, caused a decrease in *src* activity as assessed by immune complex kinase assay. The data suggest an unsuspected plasticity in the lineage commitment of adenocarcinoma of the prostate.

**K 108** BENZOQUINONE ANSAMYCINS DISPLAY POTENT CYTOTOXICITY FOR HUMAN MELANOMA CELL LINES: CORRELATION WITH MELANOCYTIC DIFFERENTIATION, Luke Whitesell, Stuart Shifrin, Charles E. Myers, Len Neckers, Clinical Pharmacology Branch, National Cancer Institute, Bethesda, MD 20892

Disseminated tumors of neural crest derivation are among the most refractory to cure by traditional chemotherapeutic regimens. Consequently, we have sought new treatment strategies based on the developmental biology of these unique neoplasms. The benzoquinone ansamycins herbimycin A (HA) and geldanamycin (GA) are known to revert the morphology of $pp60^{v\text{-}src}$ transformed fibroblasts. Because high levels of *src* protein expression and kinase activity are associated with neural differentiation, we have evaluated the effects of these drugs on the growth and differentiation of various primitive, neurally-derived tumor cell lines. Human melanoma cell lines display varying degrees of unexpected cytocidal sensitivity to these agents. Sub-micromolar concentrations result in >95% cell loss. The pattern of sensitivity appears to correlate with degree of differentiation as defined by morphology and surface markers. GA and HA are clearly useful probes to study potential tyrosine kinase interactions critical to the biology of these problematic tumors. Due to their potency, selectivity and novel mechanism of action, HA and GA may also prove clinically useful therapeutic reagents.

*T Cell Recognition of Melanoma and Tumors of the Neural Crest*

**K 200** NEW MELANOMA PROTEIN lAA335B WITH CD4 SIMILARITY, Marie E. Beckner, Mary L. Stracke, Susan M. Mackem, Henry C. Krutzsch, and Lance A. Liotta, Laboratory of Pathology, National Cancer Institute, NIH, Bethesda, MD 20892

A monoclonal antibody generated using intact A2058 human melanoma cells as antigen was used to screen an A2058 expression library. It identified a fusion protein translated from A2058 melanoma cDNA representing a new gene, lAA335B. The lAA335B cDNA has 1766 bp with a 1245 bp open reading frame predicting a protein with a MW of at least 44 kilodaltons. It has a predicted 17 amino acid transmembrane region (TMR). An immunoglobulin like domain is present on the amino terminal side of the TMR. On the carboxy terminal side of the TMR there are two possible protein kinase C phosphorylation sites. Presently, the closest match in GenBank is with CD4. Human CD4 and lAA335B are 51% identical in nucleic acid sequence and 24% identical (45% similar) in amino acid sequence.

**K 201** A CYTOTOXIC T LYMPHOCYTE (CTL) CELL LINE THAT IS RESTRICTED BY CLASS II HLA-DR15 AND RECOGNIZES A MELANOMA ANTIGEN SHARED BY AUTOLOGOUS AND ALLOGENEIC MELANOMA. Paul B. Chapman, Tohru Takahashi†, Setaluri Vijayasaradhi, Soo Young Yang and Alan N. Houghton. Immunology Program, Memorial-Sloan Kettering Cancer Center, New York, NY 10021 and †Sapporo Medical College, Sapporo, Japan.

A CTL line was established from peripheral blood mononuclear cells of a melanoma patient. Cells were cultured with irradiated autologous melanoma cells and EBV-transformed autologous B cells, in the presence of rIL-2 (20 U/ml). The CTL line was cytotoxic against autologous melanoma but not against autologous B cells or the NK target K562. Anti-CD3 monoclonal antibodies (MAb) inhibited cytotoxicity presumably because cytotoxicity was mediated through the T cell receptor. The phenotype of this CTL line, determined by flow cytometry, was $CD3^+$, $CD4^+$, $CD25^+$, $CD29^+$; CD8, CD16, CD56 were not expressed. Cytotoxicity against autologous melanoma was inhibited by MAb against HLA-DR but not by MAb against HLA-DP, HLA-DQ, or HLA class I. This suggested that cytotoxicity was class II HLA-DR-restricted. The specific HLA-DR alleles of the autologous cells were identified using both serological methods and polymerase chain reaction-amplified HLA-DR polymorphism analysis. The HLA-DR allelotype of autologous melanoma was found to be HLA-DR11, 15. The CTL line was tested for cytotoxicity against a panel of 10 allogeneic melanoma cell lines and 3 non-melanoma cell lines. Cytotoxicity by the CTL line was observed against both of the $HLA\text{-}DR15^+$ allogeneic melanoma lines; melanoma and non-melanoma lines that were DR15-negative were not killed, including a $DR11^+$ melanoma cell line. These data demonstrate that the antigen recognized by the HLA-DR15-restricted CTL line is shared by allogeneic melanoma cell lines. Experiments are currently underway to determine whether this CTL line can kill $HLA\text{-}DR15^+$ melanocytes.

**K 202** MHC RESTRICTION OF T CELL RECOGNITION OF MELANOMA ANTIGENS, Qiyuan Chen and Peter Hersey, Immunology and Oncology Unit, Royal Newcastle Hospital, Newcastle, 2300 Australia.

Previous studies have drawn attention to the frequent use of HLA-A2 as a restriction element for recognition of melanoma antigens by cytotoxic T lymphocytes (CTL). Studies were carried out on lymphocytes from 2 patients whose MHC class 1 phenotypes were A1,3 and A1,2. Studies of their cytotoxic activity against a panel of HLA typed melanoma and non-melanoma cells revealed that CD8+ CTL activity was either restricted by A1 or A3 in the first patient and A1 restricted in the second patient. There was no evidence for A2 restriction. Target cells from 5 of 7 allogeneic A1+ melanoma lines were killed by a cloned CTL from the second patient and Cold target inhibition studies suggested this was on the basis of a common antigen. Production of TNF-α from the clones paralleled their cytotoxic specificity. The biochemical nature of the antigen(s) recognized by the T cells remain under study. Cloned T cells isolated from lymph nodes were all CD4+ T cells and had cytotoxic and/or helper activity as defined by IL-2 production in response to melanoma cells. Their cytotoxic and helper activity appeared to be MHC class II DR2 restricted. Some CD4+ T cells recognized allogeneic but not autologous melanoma cells, which was taken as evidence for immunoselection in vivo.

These results indicate that antigens that are commonly expressed on melanoma cells may be recognized in the context of HLA-A1 or A3. Further biochemical characterization is needed to determine if these antigen(s) are the same as those presented in association with HLA-A2. It is also clear from these studies that MHC class II antigens may be restricting elements for naturally occurring (i.e. not generated by stimulation in vitro) CD4+ T cells with specificity for melanoma. Whether the antigen is similar or different to those recognized by CD8+ CTL remains unknown.

**K 203** FAILED IMMUNOLOGIC MEMORY AND THE RISK OF METASTASES FOLLOWING EXTIRPATION OF IMMUNOGENIC INTRAOCULAR TUMORS. Bruce R. Ksander, Y. Bando, and J.W. Streilein, Dept of Microbiology and Immunology, Univ of Miami Sch of Med, Miami, Florida 33101.

The pathobiology of metastases from ocular and cutaneous melanomas is distinctly different. From cutaneous tumors, metastases arise early after diagnosis and occur at many different sites, whereas from intraocular tumors, metastases usually develop long after the primary tumor has been excised, and locate primarily in the liver. On the presumption that immunity plays a role in determining these different patterns of tumor growth, it has been proposed that skin melanomas are only able to elicit a grossly deficient immune response which is unable to control tumor growth either at the primary site or distant metastases, whereas intraocular melanomas evoke a more effective form of partial immunity (concomitant) which, although unable to contain the primary tumor, is able to suppress metastatic spread. The eventual emergence of metastases following enucleation of the tumor containing eye implies that concomitant immunity does not have an immune memory component.

To test this possibility, we used an animal model in which minor histoincompatible P815 cells form progressively growing tumors within the immunologically privileged anterior chamber (AC) of BALB/c mouse eyes, and elicit concomitant immunity which prevents metastases. Control mice received P815 cells subconjunctivally (SCon), a non-privileged site from which the tumor is swiftly rejected. Tumor-containing eyes were enucleated 7 days after injection, and during the subsequent 8 month interval tumor-specific precursor cytotoxic T cells (pTc), IL-2, and IL-4 secreting T helper (Th) cells were analyzed quantitatively by limit dilution analysis. The results revealed that primed, P815-specific pTc were present in both AC and SCon tumor recipients within 10-14 days, and remained at comparably high frequency for the entire 8 mos. Mice with SCon tumors generated memory Th that secreted IL-2 and IL-4, which were also present throughout 8 months. By contrast, mice with AC tumors generated Th that secreted IL-2 only, and 6 months after excision of the primary tumor even these cells had disappeared from secondary lymphoid organs. We conclude that immunogenic tumors in the AC (unlike their SCon tumor counterparts) (a) fail to induce long-term memory Th cells, (b) that this failure allows concomitant immunity to subside once the primary tumor has been excised, and (c) the lack of memory renders these mice vulnerable to development of metastases thereafter.

**K 204** **AN IMMUNOGENIC PEPTIDE DERIVED BY SUBTRACTIVE CDNA CLONING RECOGNIZED BY HUMAN MELANOMA SPECIFIC T CELLS**, S. Levy, W. Harel, M. Mitchell, J. Kan-Mitchell, R. Deans, University of Southern California, Los Angeles, CA 90033

Using subtractive cDNA hybridization between human melanoma and squamous cell carcinoma, we have identified a panel of novel human genes enriched for expression in the melanoma tumor. We are currently testing products of these genes for their ability to be recognized by T-lymphocytes in cytotoxicity and proliferation assays. In addition, we are investigating the expression patterns and the products of these novel genes to elucidate their role in melanocyte differentiation and tumor progression. Based on partial gene sequence information of one of these cDNA clones, clone 50, we chemically synthesized a 17 amino acid peptide. This synthetic peptide induced proliferation of CD4+ melanoma specific tumor infiltrating lymphocytes (TILs) from melanoma patients, only when autologous EBV transformed lymphoblastoid cells are present as antigen presenting cells. A similarly synthesized peptide corresponding to a housekeeping gene saposin C, failed to induce a proliferation response in this bulk T cell population. Southern blot analysis indicates that clone 50 is a single copy human gene that hybridizes to a 6 kilobase transcript on northern blots of melanoma cell lines. In summary, we have identified an epitope of a melanoma associated antigen which is biologically active. The peptide will serve as an important reagent to stimulate proliferation of specific T cell clones and to identify the corresponding cellular target. This approach represents a method for the identification of targets for immunotherapy of melanoma.

**K 205** CHARACTERIZATION OF HNK-1 ANTIGENS IN BOWES MELANOMA CELL LINE Theo.M. Luider[1)], Sylvia Soffers[1)], Marjo J. Peters-van der Sanden[1)], Carel J.H.C. Meijers[1)], Dick Tibboel[1)], Jan C. Molenaar[1)], and Arthur W.M. van der Kamp[2)], [1)]Department of Paediatric Surgery, Erasmus University, Sophia Children's Hospital, Rotterdam, the Netherlands and [2)]MGC-department of Cell Biology and Genetics, Erasmus University, Rotterdam, the Netherlands

The neural crest is a transient ridge of cells dorso-lateral to the neural tube. Cells of the neural crest give rise to the development of a variety of organs, tissues, and cell types throughout the body. Before chicken neural crest cells colonize the developing gut two HNK-1 antigens, G-42 and G-44, are expressed which are involved in the development of the enteric nervous system. The L2/ HNK-1 family is known to be involved in cel adhesion phenomena of chicken neural crest cells. In order to search for a source for isolation of G-42 and G-44, an HNK-1 positive tumor cell line (Bowes melanoma) which originate from the neural crest was screened for G-42 and G-44. We isolated the plasma membrane HNK-1 antigens present in this tumor line and analyzed the HNK-1 antigens on 2-D immunoblots. In Bowes melanoma we found a series of HNK-1 antigens on two dimensional gels. The major proteins had a molecular weight of 110 kDa and 80 kDa, respectively. In addition we found a membrane-bound HNK-1 antigen in relative large quantities in the 42-44 kDa range, which resembles the two membrane bound HNK-1 antigens in the developing chicken gut (G-42 and G-44) in four criteria: plasma membrane-bound protein, apparent molecular weight, isoelectric point (pI 5.5), presence of the HNK-1 epitope. We investigate the possibility that Bowes melanoma cell is an accessible source for the isolation of an HNK-1 antigen which is involved in the migration and binding of neural crest cells in the developing gut.

This work is supported by the Netherlands Digestive Diseases Foundation

**K 206** TUMOR-INFILTRATING LYMPHOCYTES (TIL) FROM A MELANOMA PATIENT: SELECTIVE EXPANSION OF MHC-RESTRICTED CTL CLONES WITH IDENTICAL T CELL RECEPTORS FROM DIFFERENT MLTC CULTURES, Marialuisa Sensi, Chiara Castelli, Stefania Salvi, Arabella Mazzocchi, Roberta Mortarini, Gabriella Nicolini, Andrea Anichini and Giorgio Parmiani, Experimental Oncology D, Istituto Nazionale Tumori, 20133 Milano, Italy

TIL were isolated from a subcutaneous metastasis of melanoma and cytotoxic T cell (CTL) lines were obtained by sensitizing in vitro in mixed lymphocyte tumor cell cultures (MLTC) four separate aliquots of TILs with autologous tumor cells and recombinant IL-2. All the CTL lines were predominantly TCR $\alpha\beta$ +, CD3+, CD8+ and, although able to lyse several allogeneic targets, displayed a preferential cytotoxic activity against the autologous tumor. TCR composition was analysed by using the polymerase chain reaction with 5'V$\alpha$ or V$\beta$ specific primers and 3'C$\alpha$ or C$\beta$ primers. In fresh TILs, the entire repertoire of the V$\alpha$ and V$\beta$ gene families tested was present and no TCR oligoclonality was found in the CTL lines. Twenty-six CTL clones which exhibited CD3-dependent and MHC-restricted killing of the autologous melanoma were isolated from the four TIL cultures. TCR analysis indicated that, indipendently from the MLTC of origin, only four combination of V$\alpha$ and V$\beta$ gene families were present in the majority of these CTL clones. These V$\alpha$ and V$\beta$ gene families were not found in a panel of CTL clones which did not lyse the autologous tumor.

This study indicate that recognition of melanoma antigens strongly selects for certain types of TCR bearing T lymphocytes.

**K 207 ACQUIRED RECOGNITION OF MELANOMA PEPTIDE-PULSED B CELL LINES BY TUMOR-SPECIFIC T LYMPHOCYTES**, Walter J. Storkus*, Russell D. Salter $^{\nabla}$, and Michael T. Lotze*,$^{*}$Departments of Surgery, Molecular Genetics, and Biochemistry, and the $^{\nabla}$Department of Pathology, Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

Cytotoxic human TIL derived from melanoma lesions are typically CD3+, CD8+, TCR $^{\alpha}/_{\beta}$ + cytolytic T lymphocytes (CTL) that react with melanoma target cells in a class I-restricted, tumor-specific manner. In particular, HLA-A2-restricted CTL lines have been demonstrated to lyse autologous and semi-allogeneic HLA-A2+ melanomas, as well as fully allogeneic melanomas expressing transfected HLA-A2 molecules. These findings support TIL recognition of shared or cross-reactive melanoma antigens (peptides) presented in the context of autologous HLA-A2 class I molecules.

We are examining the range of naturally processed, HLA-A2-presented melanoma peptides in order to identify TIL-reactive sequences. We report that bulk, acid-eluted peptides (<3000 Mr) derived from HLA-A2+, but not HLA-A2-, melanomas can sensitize HLA-A2+ B cell lines (K4B and C1R.A2) to melanoma-specific TIL-mediated cytolysis. HPLC fractionation of bulk melanoma peptides will be performed to identify relevant TIL-specific peptide(s).

| | 18h-pulsed[a] | LU/$10^7$ effector cells (4h) | |
|---|---|---|---|
| Target | Peptide | TIL1074 | TIL 1128 |
| K4B | None | 12 | 4 |
| " | Mel 397 (A2-) | 21 | <4 |
| " | Mel 526 (A2+) | **88** | **75** |
| " | Mel 624 (A2+) | ND | **85** |
| Mel 526 | None | 135 | ND |
| Mel 624 | None | ND | 85 |

[a]Data representative of 2 experiments performed. Lytic values underlined are significant with $p < 0.05$.

*Molecular Biology of the Neural Crest*

**K 300** THE N-MYC/MAX HETERODIMER: REGULATION AND ROLE IN NEUROBLASTOMAS, Marc Billaud and Rene Bernards, Division of Molecular Genetics, The Cancer Center of Massachusetts General Hospital, Charlestown MA 02129.

Neuroblastoma is the most common extracranial solid tumor of childhood. The N-myc oncogene is frequently amplified in the advanced stage tumors and this event correlates with a poor prognosis. N-myc, like other myc family members, has a DNA binding domain in its C-terminal part which includes a basic region and two motifs known to be involved in dimer formation: the helix-loop-helix and the leucine zipper (bHLH/LZ). Recently the gene coding for MAX, a bHLH/LZ protein which dimerizes with myc was cloned. Heterodimer formation between c-myc and MAX allows the complex to bind on a specific DNA site whose sequence is CACGTG (CM1 site).

To understand whether the effects of N-myc in neuroblastomas are mediated through dimerization with MAX we generated N-myc and MAX proteins either by in vitro translation or as Glutathione S-transferase fusion protens in E. coli. Our data indicate that N-myc and MAX heterodimerize through the bHLH/LZ motif and bind to a CM1 site. By using a procedure combining PCR and electrophoretic mobility assay we defined from a set of completely degenerate oligonucleotides the high affinity binding site for MAX as well as for the N-myc/MAX heterodimer. MAX was found to be expressed in neuroblastoma cell lines regardless the degree of amplification of the N-myc gene. Furthermore we showed by RNase protection that the two transcripts coding for the two isoforms of MAX, which differ only by 9 amino-acids, are coexpressed in these tumors. Finally, a dominant negative mutant of MAX which lacked the basic domain required for DNA-binding was constructed and transfected in rat and human neuroblastoma cell lines that overexpressed N-myc. A number of stable transfectants were generated. The phenotype of these cells, including their metastatic properties, will be presented

**K 301** INHIBITION OF METASTATIC MELANOMA CELL GROWTH BY 12-O-TETRADECANOYL PHORBOL-13-ACETATE: REGULATION OF PHOSPHORYLATION AND SYNTHESIS OF p34$^{cdc2}$ AT G1/S BOUNDARY. Coppock, D.L., Tansey, J. and Nathanson, L. Oncology Research Laboratory, Winthrop University Hospital, Mineola, NY 11501.

In vitro growth of normal melanocytes requires addition of a phorbol ester. In contrast, TPA is growth inhibitory for metastatic melanoma cells. We have investigated the basis for this growth inhibition in the Demel melanoma cell line, established in our laboratory from a metastatic melanoma. Addition of TPA blocks the transition from G1 into S phase and from G2 into mitosis. To analyze the events of the G1 → S transition, we have synchronized the Demel cells in G1 using lovastatin for 24 hours followed by release from the block by addition of mevalonate. After 8 hours the cells begin to enter S phase. The p34$^{cdc2}$ protein kinase is thought to have a regulatory role in both the G1/S and G2/M cell cycle transitions. Using an antibody to the C-terminal peptide of p34$^{cdc2}$, we analyzed immunoprecipitations of p34$^{cdc2}$ in Demel melanoma cells. When cells are labelled with $^{32}PO_4$, p34$^{cdc2}$ was unphosphorylated in G1, was highly phosphorylated in early S and remained phosphorylated in late S and G2/M. When TPA was added at 8 hours after release from the G1 arrest, p34$^{cdc2}$ remained unphosphorylated at 16 or 24 hours. However, if TPA was added at 16 hours p34$^{cdc2}$ was phosphorylated at 24 hours. In a parallel experiment where cells were labeled with $^{35}$S-methionine, we demonstrated that the rate of synthesis of p34$^{cdc2}$ was low in G1, and increased by 24 hours. This could be blocked by addition of TPA by 8 hours but not by 16 hours. This TPA sensitive point is passed by early to mid S phase. In summary, these experiments show that the TPA sensitive checkpoint in late G1 regulates the synthesis and phosphorylation of p34$^{cdc2}$ in metastatic melanoma cells. This in turn may play an important role in regulating the entrance of these cells into S-phase.

**K 302** CHARACTERIZATION OF THE REGION OF CHROMOSOME 9p DELETED IN CUTANEOUS MALIGNANT MELANOMA. Jane W. Fountain[1], Maria Karayiorgou[1], Sharon L. Graw[1], Alan J. Buckler[1], Domenica Taruscio[2], David C. Ward[2], Marc S. Ernstoff[3], John M. Kirkwood[3], Brigitte Bouchard[4], Setaluri Vijayasaradhi[4], Alan N. Houghton[4], Nicholas C. Dracopoli[1], and David E. Housman[1]. (1) Center for Cancer Research and Dept. of Biology, M.I.T., Cambridge, MA 02139, (2) Yale Univ. School of Medicine, New Haven, CT 06510, (3) Pittsburgh Cancer Institute, Division of Medical Oncology, Univ. of Pittsburgh, Pittsburgh, PA 15213, (4) Memorial Sloan-Kettering Cancer Center, New York, NY 10021.
A region of human chromosome 9p has been identified which is frequently deleted in cutaneous malignant melanoma (CMM). Hetero- or homozygous loss of 9p21-p22 markers, including (from distal to proximal) the β-interferon (IFNB1) locus, the α-interferon (IFNA) gene cluster, c72, D9S3, and D9S19, has been detected in over 81% of CMM cell line and tumor DNAs. The putative 'melanoma' tumor suppressor gene is thought to reside near a 1.6 cM region encompassing c72 and D9S3 since two unrelated melanomas are homozygously deleted for one or both of these loci. The IFNB1 and IFNA genes still remain potential candidates for involvement in CMM since they reside close to chromosomal breakpoints in one of these two tumors and are located in a region just distal to c72. In contrast to the interferons, another 9p candidate gene, gp75, which encodes a melanoma-associated antigen, has been determined to reside outside of the c72/D9S3 region and is, therefore, believed to play an independent role in the development of CMM. Additional potential candidate genes have also been identified using the method of exon amplification on appropriate cosmid and yeast artificial chromosome (YAC) DNAs. To date, five or moreYACs have been identified from the CEPH YAC library for each of the three loci, IFNA, c72, and D9S3. These YACs are currently being used to create a more detailed physical map of this region of 9p21 and should also aid in determining the extent of the two homozygous deletions detected in CMM.

**K 303** NEURAL CREST DEVELOPMENT IN *PIEBALD* MICE
William J. Pavan and Shirley M. Tilghman
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Neural crest cells derive from the embryonic ectoderm, migrate extensively throughout the developing embryo, and differentiate into a variety of different cell types including: neurons and supporting glial cells of the sensory, sympathetic and parasympathetic nervous systems, the epinephrine producing cells in the adrenal gland, skeletal and connective tissues in the head, and pigment containing melanocytes in the dermis and epidermis. Little is known about the mechanisms directing the migration of neural crest to specific sites in the embryo, or about the factors influencing their differentiation into the spectrum of derivatives. One powerful approach to identify components involved in neural crest development has been to analyze mutant mouse strains exhibiting abnormal developmental phenotypes in neural crest derivatives.

*Piebald, (s)* mice have white spotted coats due to a complete lack of recognizable melanocytes in the white areas of the skin, and also develop megacolon due to a lack of enteric ganglion cells in the distal portion of the colon. Since both melanocytes and enteric ganglion cells are derived from the neural crest, the *s* gene product may be involved in the development of crest derivatives in the skin and colon. The *s* allele may exert its affect in several ways; including abnormal migration of neural crest cells to white regions of the skin or colon, a failure of these cells to survive, or an inability to differentiate into melanocytes and enteric ganglia. To better define the influence of the *s* loci on neural crest development, molecular markers that recognize nerual crest cells, melanoblasts and neuroblasts are being used to follow neural crest cells during development of melanocytes and enteric ganglia in the skin and gut of *s* mice.

**K 304** TRANSCRIPTIONAL REGULATION OF PKC-B IN MELANOCYTES AND MELANOMAS: IDENTIFICATION OF THE PKC-B PROMOTER, Marianne Broome Powell, Jodi Godfrey, and Richard K. Rosenberg, Arizona Cancer Center, University of Arizona, Tucson, AZ 85724
The tumor promoter, 12-O-tetradecanoyl phorbol-13-acetate (TPA), is required for prolonged growth of normal melanocytes in vitro yet it is often growth inhibitory for melanomas. Since protein kinase C is an important mediator of the effects of TPA, we are investigating the expression and regulation of PKC in melanocytes and melanoma cells. Melanocytes express PKC alpha, beta, and epsilon isotypes. The melanoma we have examined express PKC alpha and epsilon, whereas ten out of eleven melanomas do not express PKC beta. Southern analysis revealed that the gene has not been lost and that there are no gross deletions or mutations. To study regulation of the PKC beta gene at the transcriptional level, we are cloning and characterizing the PKC beta gene promoter. We have isolated five genomic clones which hybridize to a cDNA probe for the PKC beta. Restriction digests reveal that these five isolates are from two independent clones. One clone contains sequences corresponding to the two terminal exons $B_1$ and $B_2$. The other clone contains upstream sequences. We are currently mapping the transcription start site and promoter sequences. Once identified, the promoter will be fused to a CAT construct to examine whether the promoter is functional in melanocytes that express PKC B and in melanoma which do not express the PKC B.

**K 305** DOMINANT NEGATIVE AND LOSS OF FUNCTION MUTATIONS OF THE *c-KIT* (MAST/STEM CELL GROWTH FACTOR RECEPTOR) PROTO-ONCOGENE IN HUMAN PIEBALDISM, Richard A. Spritz, Lutz B. Giebel, Stuart A. Holmes, Sara Droetto and Kathleen M. Strunk. Department of Medical Genetics, University of Wisconsin, Madison, WI 53706
Piebaldism is an autosomal dominant human genetic disorder of melanocyte development characterized by congential white patches of skin and hair from which melanocytes are completely absent. A similar disorder of the mouse, "dominant white spotting" (*W*), results from mutations of the *c-Kit* proto-oncogene, which encodes the cellular receptor tyrosine kinase for the mast/stem cell growth factor. We have identified *c-Kit* gene mutations in five unrelated patients with piebaldism. One patient, who has a cytologically visible deletion of 4q12, is hemizygous for both the *c-Kit* and adjacent *PDGFRA* genes. Four other patients have point mutations within the tyrosine kinase domain of *c-Kit*. Two missense substitutions are associated with a severe piebald phenotype. Two frameshifts are associated with a variable and somewhat milder piebald phenotype. These data indicate that human piebaldism is the homologue to dominant white spotting (*W*) of the mouse, and suggest that *c-Kit* polypeptides containing missense substitutions may exert "dominant negative" effects on function of the receptor *in vivo*.

In related studies, we have utilized the polymerase chain reaction to define a repertoire of protein receptor tyrosine kinase (PRTK) mRNAs in primary human pigment cells. In addition to previously-known PRTK mRNAs, such as *c-Kit*, we have identified a number of novel PRTK mRNAs. We are currently studying the tissue distribution of these mRNAs and mapping their genes to identify potential candidate loci for other genetic disorders of pigmentation.

**K 306** EXAMINATION OF OCTAMER BINDING PROTEIN PATTERNS IN MELANOMA METASTASIS AND THE EFFECT OF DIFFERENTIATIATING AGENTS UPON MELANOMA GENE EXPRESSION. Richard A. Sturm*, Angus Thompson#, Brendan O'Sullivan#, Patrick Danoy* and Peter G. Parsons#. *Centre for Molecular Biology and Biotechnology, University of Queensland, Qld. 4072, Australia and #The Bancroft Centre, Queensland Institute for Medical Research, Bramston Terrace, Herston, Qld 4006, Australia.

It is now recognised that there is a large family of octamer binding proteins (OBPs) that appear during mammalian embryonic development in a stage or lineage specific manner and which may also be expressed in various adult organs, most notably neuronal tissue. The cellular profile of proteins interacting with the octamer control sequence (ATGCAAAT) has been examined in a number of melanoma cell lines and in secondary melanoma tissue to investigate their distribution and possible functional correlation with melanoma gene expression. Our studies have revealed two distinct melanoma OBPs that we refer to as Oct-M1 and Oct-M2. The Oct-M2 activity was seen only in cell lines derived from tumor metastases and is not present in cultures of primary melanocytes. OBP assay of a melanoma brain metastasis biopsy and a derived cell line from a familial melanoma patient showed an OBP profile consistent with that seen for human brain tissue. Melanoma lymph node metastasis has also been studied to determine the OBP profile of lymphoid invasive cells. The level of Oct-M1 activity in the pigmenting melanoma cell line MM418 was enhanced in comparison to the general Oct-1 protein when cells were treated with the depigmenting agent dithiothreitol and conversely was reduced by the differentiating and pigment inducing agents dimethyl sulfoxide and butyric acid. These differentiating agents also reduced the expression of a premelanosomal epitope B8G3 as assayed by Western blotting, and tyrosinase-related protein-1 mRNA levels as assayed by Northern blotting.

**K 307** TRANSFECTION OF PROTEIN KINASE C GENES INTO HUMAN METASTATIC MELANOMA CELL STRAINS. Douglas T. Yamanishi, Mark J. Graham, Malabika De, Barna De, Julie A. Buckmeier, and Frank L. Meyskens Jr., Clinical Cancer Center and College of Med., Dept. of Hem./Onc., U.C. Irvine, Irvine, CA 92717.

Our previous studies on the expression of the protein kinase C (PKC) isotypes ($\alpha, \beta, \gamma$ and $\varepsilon$) in human neonatal primary melanocytes and metastatic melanoma cell strains had shown that metastatic melanoma cells did not express PKC $\beta_{II}$ RNA transcripts using Northern blot analysis. We have extended our PKC studies using Northern blot analysis of fresh tumor tissues and transfection of PKC genes into metastatic melanoma cells. Expression of the PKC $\beta_{II}$ RNA transcripts were either decreased or undetectable in fresh metastatic melanoma tissues using Northern blot analysis. The role of the PKC $\beta_{II}$ gene in human melanoma cells was further analyzed by transfection. Colonies could be isolated following transfection of melanoma cells with either the selection vector alone or in combination with a PKC $\beta_I$ gene or another gene. However, transfection of melanoma cells with the PKC $\beta_{II}$ gene under a constitutive promoter induced cell death/senscence within one to two weeks. Studies are underway to express the PKC $\beta_{II}$ gene under a metallothionein promoter. These data suggest an alteration in the expression of the PKC $\beta_{II}$ gene in the progression of primary melanocytes to metastatic melanoma.

*Immune Mechanisms of Tumor Rejection and Treatment in Man and Animal Models*

**K 400** THE EFFECT OF MELANOMA-DERIVED INTERLEUKIN 6 ON MELANOMA GROWTH AND DEVELOPMENT IN A MURINE MODEL. C.A. Armstrong, S.V. Koppula, D.C. Tara, J.C. Ansel, Dermatology Service, Department of Veterans Affairs Medical Center, Portland, OR 97207

We recently reported that human melanoma cells are capable of producing a number of cytokines including interleukin 6 (IL-6). The effect of this cytokine on the growth and development of melanoma is unknown. In this study we have developed a murine model to evaluate the role of melanoma IL-6 production on the biology of this neoplasm in an immune competent syngeneic host. A B16 derived murine melanoma cell line, HFH18, was stably transfected with a pZipNeo expression vector containing a full length murine IL-6 cDNA insert. This resulted in HFH18 transfectants which expressed significant amounts of IL-6 mRNA and secreted high levels of bioactive IL-6 as determined in the B9 bioassay. IL-6 production by HFH18 cells had a profound effect on tumor growth in syngeneic C57BL/6 mice. Animals injected sc with non-IL-6 secreting cells including parental HFH18 cells, HFH18 cells transfected with vector alone, or HFH18 cells transfected with the IL-6 insert in the 3'-5' reverse orientation developed tumors that grew at a significantly more rapid rate and reached a final mean tumor volume at day 30 that was 10-fold greater than tumors that developed in animals injected with the IL-6 secreting HFH18 cells. Likewise, serum IL-6 levels were significantly elevated in animals injected with the IL-6 secreting cell lines. The median survival for animals injected with non-IL-6 secreting melanoma lines was 25 days compared to a median survival of 55 days for animals injected with IL-6 secreting cells. Thus, melanoma-derived IL-6 has a significant anti-tumor effect *in vivo*. This effect may be mediated by the ability of IL-6 to stimulate host immune effector cells or by local autocrine or paracrine effects on the growth or immunogenicity of the melanoma cells themselves. This murine model will permit detailed study of the role of melanoma-derived cytokines in the biology of this cutaneous neoplasm.

**K 401** IMMUNE RESPONSE TO MURINE MELANOMA CELLS TRANSFECTED WITH MHC GENES. P. W. Chen and H. N. Ananthaswamy. Department of Immunology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030.

Tumor-specific immunity is mediated by cytotoxic T lymphocytes that recognize peptide antigens in the context of MHC class I molecules and by T helper cells which recognize peptide antigens in the context of MHC class II molecules. To elucidate the relative importance of MHC class I and class II antigens in tumor rejection, we transfected K1735, a C3H mouse melanoma cell line that did not express either MHC class I or class II antigens, with H-2K$^k$ and/or I-A$^k$ genes and determined their tumorigenicity in normal C3H and athymic nude mice. K1735 melanoma transfectants expressing either H-2K$^k$ or A$^k$ antigens alone produced tumors in both normal C3H and nude mice, whereas most transfectants expressing both H-2K$^k$ and A$^k$ antigens were rejected in normal C3H mice, but produced tumors in nude mice. In addition, the transplantation immunity induced by K1735 transfectants expressing both H-2K$^k$ and A$^k$ antigens completely cross-protected mice against challenge with H-2K$^k$-positive transfectants, but only partially against challenge with A$^k$-positive transfectants and the parental K1735 cells. Thus, these studies demonstrate that expression of either MHC class I or class II antigen alone is insufficient to cause tumor rejection and that both are essential for the rejection murine melanoma cells. Supported by American Cancer Society grant #IM-598 to H. N. A.

**K 402** Immunity to Melanoma with IL-2-Secreting Mouse Cells Expressing Melanoma-Antigens, Cohen E.P., Kim T.S., Russell S.J., Collins M.K.L. Department of Microbiology, University of Illinois at Chicago, Chicago, IL 60612 and Chester Beatty Labs, London SW3 6JB, England Cytotoxic T lymphocytes are known to recognize an extraordinarily wide array of small peptides (in the context of MHC) including neoantigens associated with malignant cells. It may be that the growth of antigenic tumors results from a failure of immune recognition, not from the absence of tumor-associated determinants. An understanding of the cellular "defect" that enables tumor cells to grow in immunocompetent recipients could have important implications for the specific immunotherapy of cancer. As an experimental approach, we used transfection to construct IL-2-secreting, allogeneic mouse fibroblasts that express melanoma-associated antigens. We then tested the cells' immunogenic properties in terms of their ability to elicit an anti melanoma immune response in mice syngeneic with the tumor. The construct was prepared by transfecting genomic DNA from B16 cells ($H\text{-}2^b$) into LM cells ($H\text{-}2^k$) which are allogeneic with C57BL/6 mice. Colonies of transfected cells expressing melanoma-associated determinants were isolated, and then infected with an expression-competent plasmid carrying the gene for IL-2. C57BL/6 mice rejecting the IL-2-secreting, melanoma antigen-positive allogeneic mouse cells developed cellular immunity to the melanoma. This immunity exceeded that following immunization with non-IL-2-secreting constructs, or with B16 cells. Both CD8+ cells and macrophages were involved in tumor rejection. Mice immunized with the IL-2-secreting cells survived for prolonged periods following challenge with viable B16 cells. The importance of the alloantigens in the immunogen was indicated by the failure of $H\text{-}2^k$ mice to develop anti melanoma immunity following immunization with constructs that did not secrete IL-2. Thus, the highest levels of anti melanoma immunity developed in mice syngeneic with the tumor that received immunizations with IL-2-secreting cells expressing allo antigens along with melanoma associated determinants.

**K 403** EFFECT OF UV-B RADIATION ON THE METASTASIS OF MURINE MELANOMAS. Cherrie K. Donawho, and Margaret L. Kripke. Department of Immunology, University of Texas M.D. Anderson Cancer Center, Houston, TX.
Previously we reported that the outgrowth of murine melanoma cells was accelerated when they were injected into the skin of syngeneic mice exposed to UV-B (280-320 nm) radiation (UVR). We demonstrated that the phenomenon was restricted to immunogenic tumors and could not be detected in immunosuppressed mice. These results suggested that UV irradiation was impairing a local, immunological resistance mechanism that contributes to the control of tumor growth in the skin. To determine whether this immunological resistance mechanism is also important in controlling the metastatic spread of cutaneous tumors, metastatic clones of the K1735 melanoma were used in syngeneic C3H/HeN(MTV$^-$) mice. Clones 4 and M2 of K1735 metastasize spontaneously when implanted into the external ear; however, clone 4 is not detectably immunogenic, whereas clone M2 is highly immunogenic. Tumor fragments from each clone were implanted into the pinna of groups of C3H mice and excised 3 to 5 weeks later. Some mice in each group were exposed to 4.8 kJ/m$^2$ UVR twice a week for 3 weeks either before tumor implantation or during the period of local tumor growth. The mice were monitored for survival time and examined for the presence of metastases at the time of death. With the immunogenic clone M2, the average number of metastases per mouse was increased and the survival rate was decreased in the UV-irradiated groups compared to the non-irradiated mice. With the non-immunogenic clone 4, UV irradiation had no effect on the pattern of metastasis or on survival time. We conclude that UV irradiation of the skin not only facilitates the growth of local tumors, but also enhances the metastatic dissemination of cutaneous tumors; both of these effects appear to be limited to immunogenic tumors, suggesting that this effect of UV irradiation is immunologically mediated.

**K 404** REGIONAL EOSINOPHILIA CORRELATES WITH CLINICAL RESPONSE TO IL-2/LAK CELL THERAPY IN PRIMARY BRAIN TUMORS, Roberta L. Hayes, Maxim Koslow, Emile M. Hiesiger, Ellery Moore, DonnaMarie Pierz & Joseph Ransohoff, Dept Neurosurgery, NYU Medical Center, NY, NY 10016
The median survival for patients with primary, malignant glioma [grade 3/3, glioblastoma (GBM)], is approximately 56 wks following surgery, radiation and chemotherapy. After recurrence and re-operation, median survival is 26-29 wks. Twenty-seven patients with recurrent glioma were evaluated for a Phase I/II trial of lymphokine activated killer (LAK) cells and interleukin-2 (IL-2, Cetus). Post-operatively, 15 patients were eligible for therapy (2 anaplastic astrocytomas, 13 GBMs). LAK cells ($1x10^8$-$10^{10}$) and IL-2 ($1.2x10^6$-$2.4x10^6$ IU/bolus) were infused into the tumor cavity through a reservoir. Two cycles of therapy, each consisting of 6 doses of IL-2 were infused, with LAK cells given on the first day of each cycle. Unlike most brain tumor protocols, the use of corticosteroids was strictly limited during treatment. Based on survival, the 13 treated GBM patients could be divided into responder (N=7) or non-responder (N=6) groups. The overall mean survival after re-operation was 55.2 $\pm$ 3.4 wks (SEM); median 47 wks (N=13). The mean for the 7 responders, however, was 79.4 $\pm$ 12.6 wks, median 75 wks, versus a mean of 31.5 $\pm$ 1.8 wks, and a median of 29 wks for non-responders. In addition, 2/2 of the treated AA patients and 5 GBM patients are alive. The mean survival for 10 recurrent GBM patients who were re-operated on during this time period, but who were not treated with IL-2/LAK, was 28.4 $\pm$ 3.4 wks (median 26.5 wks). Prior to each IL-2 injection, fluid was aspirated for WBC differentials and cytokine analyses, (IL-2, IFN$\gamma$ and soluble IL-2 receptor). Progressive, marked eosinophilias were noted in the fluid aspirates of the responder patients, although peripheral blood eosinophil levels did not increase. Four patients had re-operations following therapy, and 2 had autopsies. The tissue nearest the catheter showed marked areas of tumor necrosis with eosinophilic and CD45R0$^+$ lymphocytic infiltrates. The chemoattraction of eosinophils to the brain is probably due to *in situ* production of IL-5 by activated lymphocytes. Persistent eosinophils and IL-2 were demonstrated in one patient by lumbar puncture 13 wks after re-operation. Supported by CA46788.

**K 405** SUCCESSFUL TREATMENT OF A MALIGNANT RAT GLIOMA WITH CYTOTOXIC T LYMPHOCYTES, Frank P. Holladay, Teresa Heitz, Yen-Ling Chen and Gary W. Wood, Departments of Surgery, Neurosurgery Division and Pathology and Oncology, The University of Kansas Medical Center, Kansas City KS 66103
Brain tumors are highly resistant to therapy. Their diffuse infiltrative nature and the relative inaccessibility of brain tissue to blood and lymph are barriers to surgical and cytotoxic treatments alike. Moreover, it is not clear whether they express antigens which stimulate immune responses in syngeneic hosts. The purpose of this study was to produce immune cells specifically reactive with an anaplastic rat glioma (RT2) and determine whether those cells could affect tumor progression in the brain. RT2-specific cytotoxic cells were prepared by priming rats *in vivo* with RT2 tumor cells and C. parvum and stimulating the primed lymphocytes *in vitro* with irradiated RT2 tumor cells and interleukin-2 (IL-2). A variety of control cell populations exhibited only low levels of cytotoxicity against various tumor target cells. Specifically stimulated cytotoxic cells exhibited a high level of cytotoxicity against RT2, but not C6, an allogeneic glioma, 3M2N, a syngeneic mammary tumor or CSE, a syngeneic fibrosarcoma, tumor cells. To generate a model for therapy, rats were injected intracerebrally (IC) with RT2, generating progressing brain tumors, which killed untreated rats in approximately two weeks. To test the therapeutic potential of the effector cells, tumor-bearing rats were treated by intravenous (IV) injection of lymphocytes on day 5 of tumor growth. Treated rats also received a five day course of systemic IL-2 beginning on day five. Treatment with IL-2 alone, RT2-primed spleen cells or RT2-primed spleen cells stimulated *in vitro* with C6 did not affect rat survival. However, tumor-bearing rats treated with RT2-stimulated lymphocytes exhibited increased survival or were cured. Systemic IL-2 was an essential adjunct, because survival was not affected by treatment with effector cells alone. Therapy initiated on day eight of tumor progression lacked effect on survival. Those results demonstrated that rapidly progressing malignant brain tumors may be treated effectively by systemic administration of tumor specific immune cells and IL-2.

K 406 PHASE I TRIAL OF INTRATUMORAL INTERLEUKIN-1 ALPHA (IL-1α). John E. Janik, John W. Smith II, Walter J. Urba, William Sharfman, Brendan Curti, Joost J. Oppenheim, Dan L. Longo. Clinical Research Branch, Biological Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute, and PRI/DynCorp., Frederick, Maryland 21701. We are conducting a phase I trial of IL-1α administered into subcutaneous and cutaneous tumors to determine the toxicity and biologic effects of IL-1 on the injected lesion. Preclinical studies indicate that intratumoral injection is the most effective route of administration to decrease tumor size and effect a cure in animals with tumors of varying immunogenicity. Patients (pts.) with advanced metastatic cancer who had at least 2 biopsiable sites of disease were eligible. IL-1α was administered intratumorally once daily for five days with doses ranging from 0.001-0.1 μg/kg to cohorts of three pts. Sixteen pts. have been treated, 13 with melanoma, 2 with colorectal cancer and 1 with renal cell cancer. The toxicity was comparable to that of intravenously administered IL-1 and consisted of fever, chills, hypotension, nausea, vomiting, headache, myalgia, arthralgia and abdominal pain. A toxicity unique to intralesional therapy was the development of acute localized erythema (56% of pts.) and two episodes of cellulitis. Melanoma biopsies (3 or 21 days post-injection) have shown no alteration in expression of class I or class II major histocompatibility antigens, or infiltration of neutrophils. One pt. had intratumoral infiltrating lymphocytes. Most pts. developed perivascular CD3+ lymphoid infiltrates in the tumor specimens or in the surrounding subcutaneous tissue. No partial or complete responses occurred. The maximum tolerated dose has not been reached.

K 407 HUMORAL IMMUNE RESPONSE IN DISEASE-FREE ADVANCED MELANOMA PATIENTS AFTER VACCINATION WITH MELANOMA-ASSOCIATED GANGLIOSIDES, Jacques Portoukalian[1], Stefan Carrel[2], Jean-François Doré[1] and Philip Rümke[3], on behalf of the EORTC Cooperative Melanoma Group. [1]Centre Léon Bérard, Lyon, France; [2]The Ludwig Institute for Cancer Research, Lausanne Branch, Lausanne, Switzerland; [3]The Netherlands Cancer Institute, Amsterdam, The Netherlands.

Several studies have shown that melanoma-associated gangliosides are immunogenic in melanoma patients and that antibodies against them have a prognostic favorable effect. Our study aimed at characterizing the humoral immune response in disease-free advanced melanoma patients vaccinated with a total ganglioside fraction extracted from pooled metastases of human melanoma, containing as major gangliosides GM3 and GD3, and as minor ones GM2 and GD2. Repeated vaccinations were done intradermally with gangliosides either in the native form in buffered solution, or in the form of liposomes. Serum samples were collected and assayed by ELISA for the presence of specific IgG and IgM antiganglioside antibodies. Out of 32 evaluable patients, 17 presented a significant increase of specific antibodies, mostly of the IgG isotype, which was maximal between two and four months after starting injections of gangliosides, and gradually disappeared within one year. Selected sera were tested by FACS analysis and immunostaining on thin-layer plates. All gangliosides seemed to be immunogenic, with somewhat more reactivity on GM2 and GD2. The disease-free intervals for the patients who showed an antibody response were significantly higher ($p<0001$) than those of the non-responding group as compared by the Kaplan-Meier method.

K 408 NEURAL CREST CELL STUDIES IN THE GENETICALLY BASED MELANOMA MODEL OF *XIPHOPHORUS*. Bahram Sadaghiani, Bruce J. Crawford*, Juergen R. Vielkind, Dept. of Path. and Anat*., University of British Columbia and Dept. of Cancer Endocrinology, B.C. Cancer Agency, Vancouver, V5Z 1L3, Canada.

*Cancer appears to result from multiple genetic changes leading to the* loss of the differentiated state of cells. With a drastic increase in the incidence of human melanoma, it is vitally important to identify the factors which regulate normal differentiation of pigment cells. In the *Xiphophorus* fish melanoma model which is well established as a model for human melanoma, the progression into the melanomatous state is accompanied by a stepwise accumulation of alterations of genetic factors mediating proper growth control and differentiation of pigment cells. The pigment cells that give rise to melanoma are derived from the transient embryonic structure, *the neural crest. In order to study normal and melanomatous* pigment cell differentiation from the very earliest stages, we have studied neural crest formation and the migration of its derivative cells in normal and abnormal genotypes in these fish. Basically, the formation of this structure as well as the migration pathways are similar to those in other vertebrates. However, the time point of neural crest cell migration appears to be genotype specific; in particular early migration was found in offspring that inherited the melanoma from the mother instead the father suggesting a maternal effect. This maternal effect may also contribute to *the earlier and greater severity of these melanomas.* Proper cell differentiation relies on both intrinsic and extrinsic signal exchange in the correct place and at the correct time. Therefore, the appearance of such ECM components as fibronectin, collagen, chondroitin sulfate, and laminin were followed by immunostaining with Abs against these components in combination with the migration pattern of neural crest cells as followed by staining with the mAb HNK-1. In general, the ECM components appeared prior/at the same time as the neural crest cells. The experiments in melanoma producers are underway as are studies in a *recently developed in vitro* system of cultured neural crest cells derived from explanted neural tubes.

K 409 A PHASE IB TRIAL OF ANTI-CD3 (OKT3) AND LOW DOSE CONTINUOUS INFUSION(CI) INTERLEUKIN-2 (IL-2) IN CANCER PATIENTS: AN APPROACH TO ENHANCE T CELL ACTIVATION, Sosman J.A., Ellis T., Bodner B., Kefer C., and Fisher R.I. Loyola University Medical Center, Maywood, IL. 60153. IL-2 based immunotherapy has shown promising anti-tumor effects in refractory malignancies. While IL-2 activates NK cells in vivo, it poorly activates T cells, which may mediate tumor effects in human cancer such as melanoma. Based upon preclinical studies which show that anti-CD3 can activate T cells, we initiated a phase I trial of escalating doses of OKT3 (10-600ug/m2) in combination with low dose CI IL-2 (2.5M IU/m2/d). Patients received OKT3 as a 5 minute IV infusion weekly for 3 doses followed 18 hours later each week with a 100 hour outpatient infusion of IL-2. Each patient also receives IL-2 alone for 3 weeks separated from the OKT3/IL-2 course by 2 weeks, in order to better define the clinical and biological effects of OKT3 upon IL-2 therapy. Patients are monitored for T cell activation in the blood and at tumor sites(if possible) by assaying: T cell expression of activation markers (CD25Ra, CD45RO/RA, HLA-DR) T cell lysis of the CD3-hybridoma, cytokine mRNA in T cells, and serum cytokine levels (IL-6, TNF). At present 13 patients with melanoma(3), kidney(6), cervical(2), and colon cancer(1), and sarcoma(1) have been entered at OKT3 dose levels of 10, 100, 200ug/m2 with accrual continuing at 400 and 600ug levels. OKT3 has been generally well tolerated with transient fever, chills, headache and hypotension. One patient developed seizures, but this was associated with rapidly developing hemorrhagic brain metastases. OKT3 has not enhanced IL-2 clinical toxicity to a significant degree. To this point, biologic studies have not revealed consistent evidence for OKT3 enhancement of T cell activation. We continue to explore additional dose levels and will present clinical and biologic data for all OKT3 doses.

**K 410** MOLECULAR AND CELLULAR STUDIES IN THE GENETICALLY BASED MELANOMA MODEL OF *XIPHOPHORUS*. Juergen R. Vielkind, Bruce Woolcock and Barbara M. Schmidt, Dept. of Path., University of British Columbia and Dept. of Cancer Endocrinology, B.C. Cancer Agency, Vancouver, V5Z 1L3, Canada.

There is an alarming increase in the incidence of human melanoma; by the year 2,000 one in 90 North Americans is expected to develop the disease. Early diagnosis is the most promising cure. However, an accurate melanoma marker is not available. The genetic *Xiphophorus* fish melanoma model is well established as a model for human melanoma. It documents the general concept of cancer, i.e. multiple genetic changes underly the progression of a normal into a metastazising tumour cell. The stages of progression produced through genetic manipulation by the researcher parallel those established for human melanoma. Two major genetic loci mediate melanoma formation in our model: the pigment pattern locus, a sex-linked complex locus encompassing information for the formation of pigment cells, their location, and permissiveness for melanoma formation, and an autosomal tumour suppressor gene controlling final differentiation. We have found two very similar, sex-linked genes, *mrk-1* and *-2*, which code for putative receptor tyrosine kinases; *mrk-2* is closely linked to the pigment pattern locus. It appears that *mrk-2* has arisen through duplication of *mrk-1* and is only present in fish which produce the pigment cells giving rise to the melanoma. mRNA expression studies using PCR revealed that the *mrk-1* is expressed in almost all tissues. *mrk-1* and *mrk-2* are expressed in the eye, skin, fins, gills, testes but *mrk-2* shows increased expression in skin where pigment cells are located. Most importantly, *mrk-2* expression increases with an increase in malignancy of the pigment cells; in malignant, metastazising melanoma only *mrk-2* expression is found. In order to study expression at the cellular level, we have generated a putative mrk-specific antibody. This antibody reacts with pigment cells from our fish melanoma cell line but not with other cell lines. Very surprisingly however, it also reacts with cells from three different human melanoma cell lines but does not show reactivity with B-cells from the same patients. Experiments are underway on sections from human melanoma tissue to test its feasiblity as a melanoma marker.

*Late Abstract*

HUMAN MELANOMA RECOGNITION BY $CD4^+$ AND $CD8^+$ TUMOR INFILTRATING LYMPHOCYTES. Francine Jotereau, Marie-Christine Pandolfino, Christophe Viret, Yannick Guilloux and Elisabeth Diez, U211 INSERM, CHR, Plateau technique, Quai Moncousu, 44035 Nantes, France.

In an attempt to better understand the spontaneous immune reactions against human melanomas we analysed the specificity, diversity and activation requirements of melanoma TIL at the clonal level. Specificity was established on the basis of specific lysis or proliferation in response to the autologous melanoma line but not to the autologous lymphoblasts, to allogeneic melanomas (not sharing an HLA antigen) or to K562. We were able to isolate $CD4^+$ as well as $CD8^+$ tumor-specific clones from the 4 metastatic melanomas studied. All these clones were strictly dependent upon the presence of autologous tumor cells but also of exogenous IL-2 and EBV-B cells for their growth. Antigen presentation by tumor cells was therefore necessary but not sufficient to induce a significant in vitro expansion of these clones. Furthermore,the $CD4^+$ clones did not produce detectable levels of IL-2, although they produced TNF, in response to a stimulation by autologous tumor cells and EBV-B cells.

On the basis of their TCR gene configuration, 5 different melanoma-specific clones (2 $CD4^+$ and 3 $CD8^+$) could be identified among 53 clones isolated from a single melanoma. Lysis by two $CD8^+$ clones appeared restricted by HLA-A2 and was partially or completely inhibited by anti CD8 antibody. Lysis by the third $CD8^+$clone was restricted by HLA-B or C antigens and was CD8 independent. The patterns of cross reactivity of the 2 A2-restricted clones with 15 allogeneic $A2^+$ melanomas suggests that they recognize 2 different peptides. These data indicate that a spontaneous and diverse immune response, mediated by tumor-specific $CD4^+$ as well as $CD8^+$ T lymphocytes, develops in most MHC bearing human melanomas but that peptide-MHC complex presentation by tumor cells is ineffective to permit a significant proliferation and therefore an optimal functioning of these lymphocytes inside the tumor.