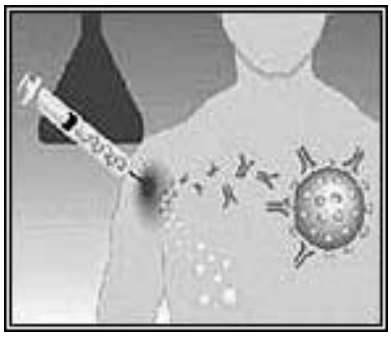

Immunotherapy for the New Century

Inmunoterapia en el Nuevo Siglo

December/Diciembre 5-8, 2002
La Habana, Cuba

Session: Innate Immune System and Its Regularory Role

Regulation of Dendritic Cell Function by Innate Stimuli

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Adaptive immune responses to foreign antigens depend largely on the ability of such antigens to activate cells of the innate immune system. For intrinsically non-immunogenic antigens such as many tumours, immunogenicity can be promoted by co-administration of an adjuvant, which activates antigen-presenting cells (APC). Many adjuvants are structures isolated from microbes, including lipopolysaccharide (LPS), bacterial DNA, bacterial lipopeptides and viral double stranded RNA (dsRNA). We have shown that these so-called pathogen associated molecular patterns (PAMPs) can directly activate murine dendritic cells (DC), prominent APC that bridge the innate and adaptive immune systems. DC activation by adjuvants results in increased presentation of antigen to T cells, up regulation of co-stimulatory molecules involved in promoting T cell expansion and, in some instances, production of cytokines that influence T cell effector development. PAMPs activate DC via pattern recognition receptors (PRRs) of the Toll-like receptor family (TLR) or via other yet-unidentified classes of PRR. Interestingly, we find that the type of PRR triggered can dictate the type of cytokine subsequently made by DC and, to a large extent, influence the subsequent class of T cell response. This requires additional signals provided to the DC by T cells, which amplify the DC cytokine response but fail to alter its quality. This suggests that molecularly-designed adjuvants that target specific DC PRR could be used in vivo to direct specific classes of immune responses to tumours.

Inducible IL-2 Production by DC Revealed by Global Gene Expression Analysis

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The immune system of vertebrate animals is characterized by the capacity to respond to perturbations without destroying self-tissues. To exert this function it uses at least three different approaches. It responds, in a few hours, to infectious agents (innate immunity) by recognizing molecular patterns typical of microorganisms and absent in self-tissues; it mounts a late response that discriminates among different microbes giving rise to memory (adaptive immunity), and, finally, it maintains tolerance against self-proteins.

Cells devoted to innate or adaptive immunity reside in non-lymphoid and lymphoid tissues where are maintained in homeostatic equilibrium. Perturbations are recognized by dendritic cells (DCs) that are key regulators in the immune system being important for the activation of a particular arm of the innate immunity (NK cells) and necessary for the priming of a long lasting adaptive immunity. Moreover, by inducing thymic deletion of self-reactive T cells and by contributing to the differentiation of regulatory T cells, they are also involved in the maintenance of self-tolerance.

The strategy of DCs to accomplish these biological effects resides in their ability to segregate in time different functions starting from the perturbation arrival. Thus, after recognition of an infectious agent, resting immature DCs undergo a maturation process that leads to a strictly defined kinetic expression of cytokines and cell surface molecules critical for activation and control of innate and adaptive immune responses. In order to identify genes involved in DC maturation, a kinetic study of immune dendritic cell transcriptome following activation with Gram-negative bacteria has been performed using microarrays representing 11000 genes and ESTs. 2951 transcripts differentially expressed during dendritic cell maturation were identified. These sequences mostly encoded enzymes, transcription factors, signal transduction molecules and proteins involved in cytoskeleton rearrangements and inflammatory responses. Among the unexpected genes, IL-2 transcript was transiently upregulated at early time points following bacterial encounter. By comparing the ability of early activated wild type and IL-2-/- DCs to stimulate alloreactive T cells, we were able to show that IL-2 represents an additional key molecule conferring T cell stimulatory capacity to DCs. The recent observation that DCs are able to produce IL-2 at early time points following bacterial interaction open interesting possibilities to explain some unique DC functional properties.

Gangliosides Purified from Human Melanoma Tumors Alter the Phenotypic and Functional Differentiation of Monocyte-derived Dendritic Cells and Induce their Apoptosis

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Gangliosides are membrane-associated sialic acid-containing glycosphingolipids which have been involved in the suppression of the anti-tumor immune response. Indeed, tumor cells synthetize and shed large amounts of gangliosides into their microenvironment and several studies have unravelled the immunosuppressive properties of these compounds. In the present study, we analyzed the effects of GM3 and GD3 ganglioside fractions purified from human melanoma tumors on the differentiation of monocyte-derived dendritic cells (MoDC). Monocytes were purified from normal peripheral blood and cultured for 6 days with GM-CSF and IL-4 in the presence or not of gangliosides at different concentrations, before phenotypic and functional studies were carried out. Results showed that gangliosides dose-dependently altered the phenotype of monocyte-derived cells: HLA-DR, CD1a, CD54, CD80 and CD40 antigens were strongly downregulated while the percentage and mean fluorescence intensity of CD86 was significantly increased. CD14 and CD83 expression remained negative. Both ganglioside fractions were effective, although, at a similar concentration (20 ?g/ml), GM3 appeared more effective than GD3. GM3 and GD3 gangliosides inhibited the allostimulatory capacity of viable monocyte-derived cells in a dose-dependent way. Furthermore, the fractions induced an increased production of prostaglandins E2 by MoDC. Since both GM3 and GD3 gangliosides altered the viable cell yield, we analysed their effects on cell apoptosis using annexin and propidium iodide staining. Results showed that both GM3 and GD3 induced MoDC apoptosis and secondary necrosis. This effect, as well as phenotypic and functional changes were reversible, however, providing the cell contact with the compounds was below 48 hours. In conclusion, the results demonstrate an inhibitory effect of gangliosides on phenotypic and functional DC differentiation, which may be an additional mechanism of human melanoma escape.

VSSP: Very Small Size Proteoliposome or Very Strong Stimulatory and Powerful Adjuvant

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Recent findings in the interactions between pathogens and the innate immune system has opened new opportunities for adjuvants and vaccine designs. We have described a new approach in the adjuvant field, in which gangliosides are incorporated into the outer membrane complex of Neisseria meningitidis to form Very Small Size Proteoliposomes (VSSP). VSSP has shown previously, a peculiar ability to render immunogenic highly tolerated gangliosides, inducing strong antibody responses, particularly Th1 related IgG isotypes. These results drove our attention to the immunopotentiatory properties of VSSP. Here we demonstrated that VSSP is as effective as complete Freund's adjuvant at inducing an antigen-specific antibody response. Moreover, given that exogenous "danger" signals can condition APC to promote Th1 or Th2 responses, we investigated the effects of VSSP on DC. Immature bone marrow DC, exposed to either LPS or VSSP, showed similar DC maturation but differences on IL12p70 and TNFa production. Also, either LPS or VSSP-matured DC promoted a Th1 response by OVA stimulated naive CD4+ T cells from DO.11.10 transgenic mice. Strikingly, VSSP effects over DC are not only related to LPS since it induced IL12p40, IL1beta and IL6 on DC from C3H/HeJ mice, which are hyporesponsive to LPS. We have also studied the effect on $CD8^+$ T cells showing that VSSP induce, on this cells, specific INFg production and CTL response to a coinjected protein. These data reveal the potent adjuvant activity if VSSP suggesting also that it belong to a class of vehicles that are able to elicit CTL responses to exogenous antigen.

Subversion of Innate-like B Cell Immunity by Microbial Proteins

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Over the last few years, our understanding of the mechanisms used by immune receptors of developing lymphocytes to sense the nature of bound ligands and to mount appropriate immune responses has made significant progress. To elude immune detection and destruction, pathogens target a variety of cellular signaling molecules and pathways, and interfere with their function. These effects may critically disturb B lymphocyte signaling, with implications for the outcome of neoplastic transformation, susceptibility to autoimmunity and infection. To investigate the effect of a microbial protein on the human immune system, we have used mice that have been engineered to express fully human antibodies. We demonstrate that the bacterial protein induces a marked deficit in lymphocytes implicated in "innate-like " B cell immunity. Normally, these latter cells are responsible for rapid clearance of pathogens and represent a first line of defence against pathogens. Thus, this work reveals a novel mechanism that may be used by some infectious agents to subvert the host's innate immune defence.

RAE-1 Recognition By NKG2D: a Molecular Mechanism For GD T-cell Mediated Surveillance of Cutaneous Malignancy in the Mouse

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T-cells execute systemic immune responses, but many curiously reside in normal peripheral tissues. We have found that local T-cells regulate tumor formation. Mice lacking either ab or gd T-cells have an increased tumor incidence following several induction regimen. This reveals neoplasia and malignant progression are strictly under immune regulation and shows non-redundant roles for systemic and local T-cells. Epidermal gd T-cells use both TCR and NKG2d to recognize carcinoma cells as cytolysis is additively inhibited by blocking these receptors. NKG2d, also on Natural Killer cells and CD8+ ab T-cells, binds the MHC Class I-like molecules, Rae-1 and/or H60, in mice and transduces an activating signal via Dap10 and/or Dap12. NKG2d ligands are upregulated in vivo during tumor induction and high Rae-1 levels associates with tumor cell clearance. gd T-cells are there for capable of providing an innate defense against tumor formation.

Metallothionein and Innate Activation of Primary Human and Mouse Monocytes

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Group IIB transition metal ions (especially mercury, cadmium, and zinc) are found in occupational and environmental settings and exert a wide range of toxicities in human tissues and organs. Although metals can be toxic to immune cells, they can also induce expression of a variety of genes (including those encoding metallothioneins [MTs]) in the absence of any discernible toxic events. The consequences of non-toxic metal exposure on immune function are largely unexplored.

Innate immune activation involves, among other events, the acute phase response and respiratory burst undergone by mononuclear cells (including monocytes), macrophages, and neutrophils, and in response to extracellular signals. Activation involves a series of events regulated by signals that must be appropriately received, transduced to the nucleus, and processed to trigger transcription and/or repression of genes. We have reported that treatment of human monocytes with both zinc, cadmium, or mercury, at levels at least tenfold lower than the minimum required to exert detectable cellular injury, significantly inhibits the ability of bacterial lipopolysaccharide (LPS) to trigger activation. We have further found that in vitro pretreatment of primary human and mouse monocytes, isolated from peripheral blood, with low, non-cytotoxic levels of $ZnCl_2$ (40 mM) or $HgCl_2$ (2mM) have significantly decreased ability to undergo activation (assessed by reactive oxygen production and interleukin-1b [IL-1b] mRNA expression in response to phorbol myristate acetate [PMA]). In addition, pre-treatment of mice with low, non-toxic levels of $HgCl_2$ (1 mmole Hg/kg body weight, i.p.) significantly inhibits the ability of monocytes isolated from peripheral blood 24 hours later to undergo LPS- or PMA-induced respiratory burst and differentiation into adherent macrophages. Finally, mice with insertionally-inactivated MT-1 and MT-2 genes (MT gene knockout mice) have a significantly lower respiratory burst, TNFá and IL-1b mRNA induction response, and phagocytic activity, after exposure to activating concentrations of phorbol ester. Collectively, these data indicate that low level exposure to inorganic zinc or mercury inhibits primary monocyte activation potential (and, therefore, innate immune function) in the absence of cytotoxicity, and that metal-induced MT is a modulator of monocyte activation. We hypothesize that environmental metals alter innate immune activity by inducing MT. MT may, in turn, modulate the activity of zinc-requiring proteins (primarily signal transduction proteins and transcription factors) essential for initiation and progression of monocyte activation. The connections among metal ion exposure, MT, and innate immune function, combined with the critical role of innate immunity in resistance to infection and tumour formation, highlight the importance of metals and metal-binding MTs in protection against these pathological events.

Supported by grants from the Canadian Institutes of Health Research, and the National Institutes of Health (US).

Programming of CD8+ T Cell Responses

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The functional development of cytotoxic CD8+ T lymphocyte (CTL) responses is guided by an instructional program set into motion during primary activation. This program governs a range of CTL functions, including primary expansion, functional differentiation, survival, and secondary expansion upon reexposure to antigen. By studying the quantitative and qualitative nature of the inductive signals received during priming, we have found that the program guiding CTL development is not invariant and can involve acquisition of some but not other functional capacities. My talk will focus on how the duration of antigenic stimulation, the nature of costimulatory signals, and the presence of CD4+ T help can influence the fate and function of CTL responses following primary activation. Data will be presented showing that the strength or duration of antigenic stimulation plays a critical role in the autonomous expansion of CTL in vivo, that T help is required for secondary but not primary expansion, and that a novel receptor for B7-1/B7-2 regulates early survival during clonal expansion of CTL.

Presentation of the Same Glycolipid by Different CD1 Molecules

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Five CD1 molecules are expressed in humans and it is unclear whether they have specialized or redundant functions. We found that sulfatide is a promiscuous CD1-binding ligand and have isolated T cell clones that are specific for sulfatide and restricted by distinct CD1 molecules. These clones have been used to compare the capacity of different CD1 to present the same glycolipid, to induce effector functions, and to form persistent immunogenic complexes. CD1a, CD1b, and CD1c molecules similarly load sulfatide on the cell surface without processing, and prime Th1 and Th2 responses. Stimulation by sulfatide-loaded CD1a persists much longer than that by CD1b and CD1c in living cells. Use of recombinant soluble CD1a confirmed the prolonged capacity to stimulate T cells. Moreover, other glycosphingolipids bind to all CD1, which suggests the presence of additional promiscuous ligands. Thus, group I CD1 molecules present an overlapping set of self-glycolipids, even though they are quite divergent from an evolutionary point of view.

Effects of Meningococcal Outer Membrane Vesicles on Mouse DCs

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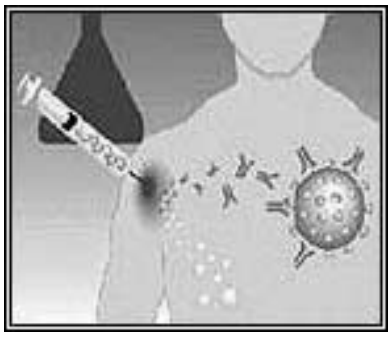
DCs are considered the most potent APCs, with the unique capacity of inducing primary immune response. Phenotype and physiology of these cells change from an immature to a mature state directed by endogenous and exogenous so-called ´´danger signals´´, including head shock proteins, infectious virus, microbial pathogens and bacterial antigens. The maturation process increases expression of costimulatory and antigen presenting molecules, and promotes cytokines and chemokines secretion, providing critical signals for lymphocyte development and differentiation. In this study we evaluated the capacity of Outer Membrane Vesicles (OMVs) obtained from Neisseria meningitides to induce DCs maturation and their ability to enhance antigen specific CTL response. OMVs were able to increase levels of costimulatory and antigen presenting molecules expression. This effect was observed even in the presence of polimixin B, suggesting that not only LPS, but also proteins contained in OMVs are playing a role as maturation inducers. OMVs enhanced the endocytic activity of murine DCs. Evaluation of cytokines profile indicated similar capacities of OMVs and LPS to promote IL12 secretion on mature DCs. However, OMVs compared with LPS, increased 2 times the percentage of DCs secreting TNF alpha and also the quantity of cytokine produced. DCs maturated in the presence of OMVs promoted naïve CD4+ T cells proliferation, and increased IFN gamma secretion on naïve CD8+ T cell. In C57bl/6 mice treated with OVA and OMVs, it was detected by ex-vivo tetramer staining an increased frequency of CD8+ T cells specific to SIINFEKL OVA peptide. The use of OMVs as adyuvant also provide an increase in CTL specific response against OVA peptide pulsed MC576 cells.



Immunotherapy for the New Century

Inmunoterapia en el Nuevo Siglo

December/Diciembre 5-8, 2002
La Habana, Cuba

Session: Cancer Vaccines

Gene Therapy for Head and Neck Cancer using Vaccinia Virus Expressing Interleukin-2

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We evaluated the efficiency of a replication competent, attenuated recombinant vaccinia virus (rvv) expressing interleukin-2 (rvv-IL-2) as a tumor vaccine in an immuno competent murine model of head and neck squamous cell carcinoma (HNSCC). These tumor cells (SCC VII/SF) have the confirmed tumor histologic characteristics of squmous cell carcinoma, the most common tumor (~90%) in the head and neck region. The tumors also resemble the biological behavior of tumor progression seen in human HNSCC. Thus, results with this model are likely to be translated for development of therapy of HNSCC patients. Tumors were generated by injection of SCC VII/SF cells in the floor of the mouth (FOM) of the syngeneic C3H/HeJ mice. When tumors were ~40 mm^3 (5-7 days) treatment was started by the intratumoral injection of rvv-IL-2. Vaccine efficiency was evaluated by the determination of survival of the mice and the tumor size. Mice treated with rvv-IL-2 survived significantly longer ($P<.03$) compared to groups of mice treated with control rvv expressing â-galactosidase or those that were mock vaccinated. However, tumor regression was not achieved by this treatment. Depressed cell-mediated immunity is a frequent event in HNSCC patients and is characterized by impairment of T cell proliferative responses. To determine the presence of similar immune suppression in this murine model caused by the tumor, proliferation of T cells was assayed in the presence of the T cell mitogen Concanavalin A (Con A). Con A induced proliferation of splenocytes, bone marrow and lymph node cells of the mice bearing tumor in the FOM was significantly lower compared to the naïve mice as well as mice bearing subcutaneous tumors. In order to circumvent tumor-induced immune suppression and thereby improve the vaccine efficiency, mice were immunized subcutaneously with irradiated, rvv-IL-2 infected tumor cells prior to or along with intratumoral vaccination. Mice treated by this protocol survived longer compared to those treated with intratumoral vaccination alone. Moreover, tumor growth was significantly inhibited ($P<.0002$) and tumor regression was observed in all mice. To study the role of various immune cells in anti-tumoral activity, numbers of CD4+ and CD8+ lymphocytes as well as macrophages were determined in the tumor beds by immunohistochemistry and in tumor draining lymph nodes by flow cytometry. Significant increase in the number of macrophages (two-fold) was observed in the tumor bed and lymph nodes of mice treated by subcutaneous vaccination along with intratumoral vaccination compared to those treated by intratumoral vaccination alone. It is concluded that subcutaneous vaccination along with intratumor vaccination improves anti-tumoral immunity of tumor bearing mice and macrophages may have role in this anti-tumor immunity.

β-receptor of IL-2 May Be Involved in Overexpression of Restriction Factors in Human Cancers

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Background: Antibodies can kill tumor cells by complement dependent cytotoxicity (CDC). CDC is implicated in antibody-mediated passive immunotherapy and antibody-inducing active specific Immunotherapy, which are employed to target tumor associated antigens. Human squamous cell carcinoma (SCC) expresses tumor associated target antigens, such as cell surface-associated sialyl Lewis antigens. Tumor cells escape CDC by expressing complement restriction factors (CRFs), namely CD59, CD46 and CD55. SCC cells overexpress CRFs. The causal factors responsible for CRF-overexpression in cancer cells are not known. An understanding of the factors regulating the expression of CRFs will be useful to improve immunotherapy involving CDC.

Hypothesis & Experimental Strategy: We hypothesized that cytokines associated with anti-tumor activities may regulate the expression of CRFs. Our hypothesis gains support from the report that the peptide sequence of one of the CRFs (CD55) may share homology with a sequence found in IL2-receptor. Further, recombinant IL2 is employed as biotherapeutic anti-tumor agent against solid tumors. Therefore influence of rIL2 on CRF-expression deserves attention. To examine our hypothesis, we have tested the response to rIL2 on SCC expression of CRFs. Using SCC71 cell line as our model, we have tested the effect of serially diluted rIL2 on the expression of CRFs. Expression of CRFs were monitored by cell suspension ELISA (Cs-ELISA) and Flow cytometry using Mouse anti-human CRF antibodies with their respective isotype controls.

Results: Results of Cs-ELISA showed that rIL2 dosimetrically augmented the expression of CD59, CD46 and CD55, with a peak at 80 ng/ml. Flow cytometric observations confirmed the above results. To determine whether rIL2 binds to its receptor, we used two types of antagonists, competitive and non-competitive antibodies. The competitive antibody blocks IL2 binding to the b receptor and also lowered the expression of CRFs induced by IL2. The non-competitive antibody bound to another region of the IL2-b receptor, not recognized by IL2 but enhanced expression of CRFs with or without IL-2. To further document that IL2 binds to its receptors on SCC 71, we studied the signal transduction via the IL2 receptors. The b subunit of the IL-2 receptor within the cytoplasm is complexed with series of kinases. The unique kinase that is associated with the IL2 b receptor is Stat-5. Once IL2 binds to its receptor Stat-5 becomes phosphorylated. Using Westernblot analysis, the changes in the levels of Stat-5 and Phospho-Stat-5 were observed during different time intervals after the addition of IL2. Interestingly the level of stat-5 decreases within 15 minutes after adding IL-2 and gradually rises to its normal level (before activation) suggesting that levels of Stat-5 gets phosphorylated. Whereas phosphorylated Stat-5 levels increase within 15 minutes and gradually decrease to its normal level.

Conclusion: IL-2 may enhance the expression of CRFs. Stat-5 kinases are implicated in the IL2-induced augmentation of the CRFs in SCC-71.

A Synthetic Peptide Homologous to the Functional Domain of Human Interleukin 10 Downregulates the Expression of the MHC Class I Alpha Chain and Tap 1/2 in Human Melanoma Cells

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Tumor cells treated with IL-10 were shown to have decreased but peptide inducible expression of MHC class I, decreased sensitivity to MHC class I restricted CTL and increased NK sensitivity. These findings could be explained, at least partially, by a down-regulation of TAP1/TAP2 expression. Here IT9302, a nanomeric peptide (AYMTMKIRN), homologous to the C-terminal of the human IL-10 sequence, was demonstrated to mimic these previously described effects of this cytokine on MHC class I related molecules and functions. We observed a dose dependent down-regulation of MHC class I at the cell surface of melanoma cells after 48 hours treatment with IT9302. In line with these results, we also observed a 40 to 50% inhibition in the expression of both MHC class I alpha chain and TAP-2 in peptide treated cells as measured by RT-PCR. A dose dependent inhibition by IT9302 of the IFN-γ mediated induction of MHC class I heavy chain and TAP1 and TAP2 proteins was also found using Western blot. Peptide-treated melanoma cells were shown to be more sensitive to lysis by NK cells in a dose dependent way. Taken together, these results demonstrate that a small synthetic peptide derived from IL-10 can mimic the antigen presentation related effects mediated by the cytokine, such as down-regulation of MHC class I and TAP1 and TAP2 expression, in human melanomas and affect tumor sensitivity to NK cells. The *in vivo* relevance of these effects in tumor immunogenicity are currently being investigated using murine models.

FONDECYT 1000888

Mimotopes of Tumor Associated Carbohydrate Antigens for Immune Therapy

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Aim: To determine if immunization with a peptide mimic of a tumor associated carbohydrate (TAC) antigen could augment immune responses to tumor cells, a peptide mimic of neolactoseries Lewis Y(LeY) and extended sialylated Lewis x (sLex) antigens expressed on human adenocarcinomas was evaluated in a murine tumor model.

Methods: Groups of mice (8-12 mice/group) were inoculated in the right-flank with 5 x 10^5 sLex expressing Meth A cells. Seven days later, separate groups were immunized with 1- peptide mimic; 2- a combination of IL-12 and peptide; 3- a combination of IL-12 and control peptide. We immunized a separate group with DNA encoded mimotope plasmid, and later challenged with Meth A cells.

Results: We observed that the peptide mimetic mediated high T-cell proliferation compared to controls. Analysis of the cytokine profile indicated T-cell activation with IFN-γ production, with no production of IL-4 and IL-10- Proliferation was APC dependent and inhibitable by anti-Class II and anti-Class I antibody. Peptide mimotope immunization of Meth A cell-primed animals enhanced anti-Meth A CTL activity. Peptide immunization alone moderately affected tumor growth as we saw tumor regression in five out of twelve mice. IL-12 significantly enhanced anti-tumor effect of peptide as tumor was eradicated in all mice, treated with peptide/IL-12. Control peptide did not affect tumor growth in combination with IL-12. DNA immunization with peptide mimic encoded plasmid significantly suppressed tumor growth (only 1 mouse out of 8 developed tumor) compared with the peptide control (7 mice out of 8 developed tumor).

Conclusions: Peptide mimotopes of TAC offers a novel strategy to mediate responses to tumor cells.

Suppression of Tumor Growth by Novel Peptides Homing to Angiogenic Vessels Application of Phage Peptide Library

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In vivo selection of peptides having affinity to a target tissue, phage peptide library is a useful technology. On the other hand, cancer chemotherapy targeted to angiogenic vessels is expected to cause indirect tumor regression through the damage of the neovasculature without the induction of drug resistance. To develop a neovasculature-specific drug delivery system, we isolated novel peptides homing to angiogenic vessels formed by a dorsal air sac method from a phage-displayed 15mer peptide library. Three distinct phage clones selected by the biopanning prensented following amino acid sequences, PRPGAPLAGSWPGTS, DRWRPALPVVLFPLH, or ASSSYPLIHWRPWAR and they showed the marked accumulation in murine tumor xenografts, respectively. The truncated peptides were synthesized from these peptides and we found that the pentamer sequences containing WRP or PRP showed similar inhibitory activity against the accumulation of the selected phage clones to the tumor tissue. Then we synthesized C18-acylated pentamer peptides and prepared cholesterol-lecithin liposomes containing the C18-pentamer peptides. Liposome modified with C18-APRPG showed high accumulation in murine tumor xenografts, and APRPG-modified liposome encapsulating Adriamycin effectively suppressed experimental tumor growth. Next, we investigated whether the peptides selected in murine angiogenic model have affinity for angiogenic endothelium derived from human tissues. Confocal observation demonstrated that the APRPG-modified liposome specifically bind to the human umbilical vein endothelial cells (HUVEC) only when the cells are stimulated with VEGF. Furthermore, histochemical analysis demonstrated that biotinylated PRP-containing peptide specifically bound to angiogenic endothelium in human tumor tissues. These data indicate that PRP-containing peptides may be useful for human cancer treatment.

The Optimisation of Clinical Cancer Vaccines; from the Laboratory to the Clinic

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We have previously reported that allogeneic cells are inherently immunogenic and can induce effective protection in the presence of shared antigens. We now report that whereas cytokine transfection (IL-2,4,7,GM-CSF) of autologous cells geatly enhances their immunogenicity, little benefit is gained when this is performed on allogeneic cells. A large number of adjuvants have been screened and few are effective at enhancing the allogeneic response. However, one of the most effective to date is a thalidomide analoque c-5013 which not only enhances anti tumor responses to allogeneic cells but also induces a strong memory response. Of note is the extraordinary anti-angiogeneic properties of this and similar compounds, two of which are already in clinical trials where their immunostimulatory properties have been confirmed.

In order to enhance anti tumour responses we have added allogeneic cells to dendritic cells both as a pulse as well as cell fusions. We have now perfected a freeze thaw process that actually ENHANCES antigen presenting function which allows us to prepare multiple vaccine samples from one bleed. We have just finished a phase I/II study on patients with prostate cancer and the results will be presented. Studies on a range of tumors are being planned based on the results of this trial and ongoing laboratory models.

Evidence for IgM Response to GD1a and GT1b in Patients with Early Stage Prostate Carcinoma and Melanoma

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Using specific monoclonal antibodies to measure cell surface density of different gangliosides in human prostate carcinoma cell lines with ELISA system, we have examined the expression of gangliosides in five prostate cancer cell lines. Most of the cell lines expressed high density of GM2, GD1a, GD1b and GT1b on the tumor cell surface. We have screened the sera of 14 prostate cancer patients (TNM stage T1c) for antibodies against GM3, GM2, GM1, GD3, GD2, GD1b, GD1a and GT1b using a sensitive ELISA. None of the patients showed IgG antibodies to any of the gangliosides. While the IgM titers of GD1a and GT1b were very high and ranged between 400 and 6400, the IgM titers against other gangliosides remained low, suggesting that the major prostate carcinoma-associated gangliosides GD1a and GT1b signaled antibody production in these patients. Although GM2 and GD1b are found on tumor cell surface, the serum titers against these gangliosides were low in most of the patients. Sera of Stage III melanoma patients were used as positive controls, which showed high titers of IgM to GD3, GD2, GM2 and GD1b.

While IgG antibodies to gangliosides are low or negligible, the profiles of IgM antibodies are most prevalent in the sera of melanoma patients (TNM stages T1a/b & T2a/b). The titers were high only for anti-GD1a and anti-GT1b IgM antibodies. Although GD1a and GT1b were reported in melanoma tumor biopsies and cell lines, the antibody response in early stage of the disease was intriguing. We have compared the serum anti-GD1a and GT1b IgM titers of the patients who had recurrent disease and expired (EXP) after surgical resection of the primary (median survival time 23.9 months) with those who are alive and have no evidence of disease (NED) (median follow up time 203.4 months) within 6 months after surgery. We found that the titers of anti-GD1a IgM ($p < 0.01$) and anti-GT1b IgM ($p< 0.01$) were significantly higher in patients who expired due to recurrent disease as compared to those with NED, suggesting that anti-GD1a and anti-GT1b IgM antibodies are poor prognosticators of stage T1 &T2 melanoma with or without ulceration.

In this study, we have identified, for the first time, the gangliosides GD1a and GT1b as important immunogenic gangliosides of early stages of prostate cancer and melanoma. GD1a and GT1b are known as potent immunosuppressive gangliosides, the former induces production of IL-10. Probably, IgM antibodies are produced during early stages of the disease to clear them from circulation to prevent immunosuppression. Supported by Defense Prostate Cancer Research Program (Grant No: DAMD17-01-1-0062) of the U.S. Army Medical Research and Materiel Command of CDMRP and State of California, Department of Health Services (TTP1020) for Melanoma Research.

Acetylated and Glycolylated GM3 Cancer Vaccines: What We Have Learnt?

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Therapeutic cancer vaccines have the potential to change the standard of care of cancer patients. At the present time basic and clinical research is centered on developing therapeutic cancer vaccines for patients who already have cancer. Although 3 cancer vaccines are currently marketed for very advanced colon cancer and melanoma patients (with very modest outcomes) the rest of the more advanced vaccines projects have achieved big failures. The presence of substantial amounts of GM3 ganglioside and its derivatives on human melanomas and other tumors, together with its peculiar biological properties, make these glycolipids unique targets for cancer immunotherapy. B16 mouse melanoma expresses GM3 and then constitutes a unique model for the development of novel GM3-based vaccines. The vaccine formulations were based in the incorporation of purified GM3 or NGcGM3 into the outer membrane protein complex from Neisseria meningitidis to form very small size proteoliposomes (VSSP). In the present work we show how pre-clinical and clinical data suggested the dependence of tumor outcome with some critical parameters associated with tolerance and tumor escape mechanisms. Also first evidences about the cellular immune response dependence of the antitumor protection of the GM3/VSSP vaccine in MB16 model are provided.

Tumor Cells as Active Specific Immunotherapy of Patients with Metastatic Cancer

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Several different strategies have been explored to up-regulate tumor-specific immunity in order to reduce the rate of recurrence in patients following surgical excision of the tumor. Our group is interested in autologous and allogeneic (for those patient in whom autologous cell line could not be established) tumor cell-based vaccines for metastatic cancer. From resected metastatic tumors, we established short-term cell cultures for use as autologous tumor cell vaccines. Following a baseline test of delayed type hypersensitivity (DTH) to an i.d. injection of 10^6 irradiated autologous tumor cells, patients received 3 weekly s.c. injections of 10^7 cells, had a repeat DTH test at week 4, and then received monthly vaccinations for 5 months. A positive DTH was defined as =10 mm induration; survival was determined from the first DTH test. In vitro proliferation and cytotoxicity of pre- and post-vaccination lymphocytes were determined to measure the immune response elicited by the vaccine. We successfully established autologous melanoma cell lines in 299/695 patients. There were 142 patients treated, and 125 had a baseline tumor DTH test recorded. Only 17/125 (14%) were tumor DTH positive at base line. Tumor DTH converted to positive in 31/80 (39%). At a median follow up >5 years, survival was better for patients whose tumor DTH converted to positive compared to patients who were DTH positive at baseline (median 37.5% vs. 11.9 months, $P_2 = 0.07$). The patient-specific, cell culture-derived, autologous tumor cell vaccine induced anti-tumor immune reactivity, manifest by tumor DTH skin testing and lymphocyte function, that was associated with improved survival in patients with advanced cancer. While these results suggest that there is a benefit, we reasoned that the coordinate engagement of tumor cells-pulsed professional antigen-presenting cells (dendritic cells, DC) with cell-mediated effector cells in vivo may be vital for augmenting effective anti-tumor responses. We have successfully initiated a trial of autologous monocyte derived-dendritic cells (DC) loaded with autologous tumor cells (TC; Melanoma and Renal Cell Carcinoma) that have been cultured with interferon-g to up-regulate tumor-associated antigens. No significant toxicities were observed in the first 6 patients. Induction of an immune response to autologous TC was confirmed by DTH test in two patients. Accrual to tumor cell-loaded DC vaccine trial continues. We are exploring allogeneic TC vaccines in patients for whom autologous TC lines are unavailable.

Immunization with Dendritic Cells Generated in the Presence of Gm-Csf and IL-13 Loaded with an Allogeneic Melanoma Cell Lysate: A Phase I Study in Patients with Metastatic Melanoma

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Objective: The objective of this study was to evaluate the safety of immunizations with autologous D pulsed with a lysate derived from the allogeneic melanoma cell line M17 in patients with advanced melanoma, and to assess the immune and clinical responses induced after vaccination. We have standardized a clinically compatible process to generate a large quantity of monocyte-derived dendritic cells referred to as Dendritophages (D^{TM}) which are differentiated in serum-free medium containing GM-CSF and IL-13.

Design and methods: Sixteen stage III or IV melanoma patients were included in the study. Vaccinations were performed at days 0, 22, 43 and 85 with an average of 4.4×10^7 D, injected subcutaneously, intradermally and intranodally. Half of the cells were loaded with a tumor cell lysate and half with the control antigens hepatitis B surface protein and tetanus toxoid.

Results: No severe adverse events related to the vaccination were observed. Preliminary results have shown the presence of specific immune responses against both control and tumor antigens in treated patients after vaccination. These responses have been detected ex-vivo in peripheral blood by IFN-ELISPOT. Objective clinical responses have been observed after completion of the treatment in 2 out of 8 evaluable patients. One patient showed regression of in-transit metastases four months after their last vaccination leading to a complete response. Another patient showed stabilization of lung metastases and remained in stable disease for four months.

Conclusion: These preliminary results indicate that this type of vaccination is safe and suggest that vaccination with D loaded with an allogeneic melanoma cell lysate may induce tumor specific immune responses and have an anti-tumor effect *in vivo*.

Study financed by an EU grant. Contract BIO4-97-2216 "Cellular Vaccines".

Epidermal Growth Factor Based Cancer Vaccine for Non-Small Cell Lung Cancer Therapy: Report from a Phase I Scale Up Trial

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Epidermal growth factor (EGF) plays an important role in the regulation of tumor growth upon binding to the EGF receptor (EGFR). Several strategies had been developed to disrupt the EGFR associated signal transduction cascade. Therapeutic approaches include monoclonal antibodies and small molecule tyrosine kinase inhibitors. Our method consists of active immunotherapy with human recombinant EGF (hu-EGF) intended to induce EGF immune deprivation.

Previous studies have demonstrated that vaccination with EGF is immunogenic and safe in 70 advanced cancer patients. In 40 Non-small cell lung cancer (NSCLC) patients, those who developed a high antibody response had a significant increase in survival in comparison with bad responders. To optimize the immunization scheme, a phase I trial was designed to evaluate the effect of vaccine dose escalation on immunogenicity and survival. Twenty advanced NSCLC patients were randomized to receive 70 mg (single dose) or 140 mg (double dose) of hu-EGF, coupled to a carrier protein and adjuvanted in Alum. Thirteen patients (65%) developed antibody titers against the EGF, defined at least 4 times above baseline. In the double dose group, 8 patients (80%) achieved seroconversion, while 5 patients (50%) developed anti-EGF antibody titers in the single dose arm. The geometric mean of the antibody titer was higher in the double dose group. No significant toxicity was seen after vaccination. Main adverse events consisted in chills, fever, nausea, vomiting and cephalea. As a surrogate endpoint of vaccination, EGF concentration was quantitated in sera using a commercial kit. After immunization, a statistically significant inverse correlation between antibody titers and EGF concentration was evidenced. Double dose treated patients showed a trend to increase in survival in comparison with the single dose immunized subjects. Patients with high antibody titers had a significant increase in survival compared with bad responders or a historical control group.

Current Status of Cancer Vaccines Against Cell Surface Antigens on Small Cell Lung Cancer

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We have identified a series of antigens that are expressed abundantly at the cell surface of SCLC and are excellent targets for immune attack. The optimal approach for inducing antibodies against each of these carbohydrate antigens has been chemical conjugation to KLH and mixture with a saponin immunological adjuvant such as QS-21. Using this vaccine approach, we have induced antibodies in the majority of patients against the following carbohydrate antigens and tumor cells expressing these antigens: glycolipids GM2, GD2, GD3, fucosyl GM1 and globo H, and the embryonic NCAM antigen polysialic acid. The GM2, fucosyl GM1 and globo H conjugate vaccines proved optimally immunogenic without further modifications, but the other antigens required chemical modifications to increase their relevant immunogenicity. This included formation of lactones of GD2 and GD3, and N-propionylation of polysialic acid.

Studies in mice indicate that passively administered or vaccine-induced antibodies against these antigens can eliminate circulating tumor cells and micrometastasis, i.e. minimal residual disease. While a randomized Phase III trial with the GM2 ganglioside vaccine in melanoma patients has proved negative to date, and a Phase III randomized trial with sTn-KLH vaccine in breast cancer patients is currently ongoing, our focus is on the development of polyvalent cancer vaccines designed to overcome the heterogeneity inherent in cancer cells and in the human immune response. In preparation for these (and new since the CIM meeting of November 2000) we have tested a new semi-synthetic saponin immunological adjuvant, GPI-0100, which appears to be at least as potent an immunological adjuvant as QS-21 and less toxic, and succeeded in inducing antibodies against GD2 and poly-a2, 8-sialic acid (expressed on SCLC and serogroup B meningococci) by modifying the antigens as described above. Results of our completed clinical trials with GPI-0100, glycolipid and polysialic acid vaccines as well as of our ongoing trial with KSA (EpCAM) vaccines will be reviewed.

Fusion Proteins Between HTGFA or HEGF and p64k Protein from Neisseria meningitidis *as a New Therapeutic Cancer Vaccine Candidate*

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The Epidermal Growth Factor Receptor (EGFR) family and its peptide ligands are involved in normal and neoplastic development and many of them are over-expressed in human carcinomas as compared with their normal counterparts.

Two recombinant fusion proteins between hEGF or hTGFa and P64k protein were developed as putative vaccines for epithelial tumor therapy. The immunogenicity of these proteins in different adjuvants was characterized in a mouse model. All immunogens were effective for the generation of specific humoral responses against growth factors. Additionally, hEGF-fusion protein vaccines induced anti-murine EGF antibody levels similar to those obtained against hTGFa in mice immunized with the other fusion protein. Oily adjuvants were superior to alum in terms of anti-hEGF/ hTGFa serum antibody titers and specific IgG secreting B cells in lymph nodes (LNs). Besides, increased levels of IgG2a and IgG2b antibodies were observed in the case of formulations containing the oily adjuvants. The inmunodominant parts of hEGF-fusion protein are the B-loop and the C-loop/C-terminal region, while in the hTGFa-fusion protein is the C-loop/C-terminal region only. Those regions include key residues for EGF or TGFa binding to the EGFR that suggest the generation of neutralizing antibodies. Remarkably the anti-hTGFa serum recognized the natural human TGFa precursor present in cell lines

A New Approach for Cancer Immunotherapy: A Vaccine Based in the Extracellular Domain of the Epidermal Growth Factor Receptor

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The epidermal growth factor receptor (EGFR) plays a central role in the development and progression of many tumors and its overexpression is associated with decreased survival, being an attractive target for cancer treatment. We introduce here a new therapeutic cancer vaccine based in the extracellular domain of human EGFR (Her1-ECD). Vaccination of mice with Her1-ECD in appropriated adjuvants induced both specific IgG secreting B cells and CD4+ T cells. The high titer specific antibody response induced by the vaccination showed IgG2a and IgG2b isotypes, which are related which a TH1 pattern. Besides, sera from immunized mice reacted with antigen-positive cell lines and inhibited the EGF/EGFR binding. Furthermore, to test our concept of autologous vaccination, mice were immunized with murine EGFR ECD (mEGFR-ECD) in appropriated adjuvants, and a high titer specific antibody response was also induced with a TH1 associated isotype profile and a DTH response was obtained. No evident toxicity associated to vaccine administration was detected.

Immunotherapy of Metastatic Melanoma with an Heterophilic Ganglioside Cancer Vaccine (NEUGCGM3/VSSP)

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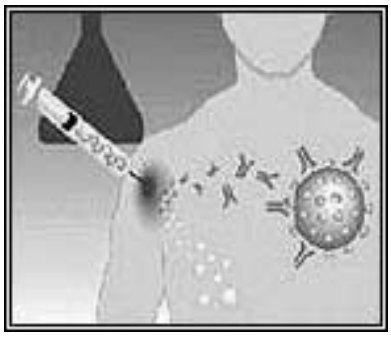
A previous clinical trial with the heterophilic ganglioside NGcGM3/VSSP/Montanide ISA 51 vaccines showed to be safe and immunogenic in advanced breast cancer patients (Proceedings 36th ASCO meeting). Now, for the first time, this cancer vaccine, obtained by combining NeuGcGM3 with the outer membrane protein complex of Neisseria meningitidis to form very small size proteoliposomes (VSSP), was tested in a Phase Ib clinical trial in stage IV melanoma patients. 10 patients have been included to receive 9 im (0,2 mg) doses. The first 5 doses (induction phase) were given at two weeks intervals, while the remaining of the treatment was given monthly. Serum anti-NGcGM3 antibody levels and DTH vs the vaccine complex were monitored during the trial. 56 days after the first inoculation nine patients' sera (one patient didn't complete the induction phase) contained specific IgM (range 640 to 2560) and IgG (range 80 to 2560). Anti-ganglioside IgA was detected in most patients sera. No evidences of serious or unexpected adverse effects have been observed. Main toxicities included erythema, mild local pain, and low grade fever. Vomiting and chills were also reported. It was encouraging to observe objective responses in 3 patients. In two of these patients regression of cutaneous metastases and stabilization of lung lesions for more than 18 months were documented. The other patient showed a mixed response with elimination, stabilization or progression of cutaneous tumors. Noteworthy vitiligo was developed by 4 patients during the vaccination period. These preliminary clinical evidences of the NGcGM3/VSSP/Montanide ISA 51 vaccine effectiveness in stage IV (without curative surgical resection) melanoma patients, although in few patients, are rather stimulating and deserve further investigation.



Immunotherapy for the New Century

Inmunoterapia en el Nuevo Siglo

December/Diciembre 5-8, 2002
La Habana, Cuba

Session: Dominant Tolerance and Autoimmunity

Pathways to Tolerance in Autoimmunity

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T cell-mediated autoimmune disease characterized by an inappropriate activation, expansion and destruction of self-tissue. Recent data has suggested that immunotherapies targeted at the regulation of peripheral T cell tolerance can impact the incidence and progression of disease. This presentation will focus on recent developments in the understanding of peripheral regulation of immunity. Emphasis will be placed on the identification and analysis of regulatory T cells that suppress pathogenic T cell activity, the role of CTLA-4 and Notch-1 in controlling T cell function and recent efforts to develop therapeutics that target autoreactive T cells. Results of studies using novel TCR-specific agents that enhance immune regulation and can prevent diabetes in animal models will be discussed. Finally, results will be presented demonstrating that these general principles can be applied to the clinical setting in diabetes.

T-cell Therapeutic Vaccines Against Autoimmune Diseases

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Vaccines are prophylactic in the sense that they are administered to healthy individuals to prevent a disease. Nevertheless, there is a growing trend to use vaccines to alleviate the suffering of those already with a disease. For every autoimmune disease for which there is a putative molecule responsible for the disease, it should be possible to produce an analog that could serve as a therapeutic vaccine. We have developed such a vaccine against multiple sclerosis (MS), copolymer 1, which is by now used as a treatment by tens of thousands of patients. A similar approach will be described also for myasthenia gravis (MG).

Copolymer 1 (Cop 1, glatiramer acetate, Copaxone) is a synthetic amino acid random copolymer, composed of L-alanine, L-glutamic acid, L-lysine and L-tyrosine. Cop 1 was designed to simulate myelin basic protein (MBP), one of the major myelin-derived autoantigens implicated in the pathogenesis of MS, which induces experimental autoimmune encephalomyelitis (EAE) - an animal model of MS. Cop 1 was shown to suppress EAE induced by MBP in guinea pigs, rabbits, mice, rhesus monkeys and baboons. The suppressive effect of Cop 1 in EAE is not restricted to a certain species, disease type or encephalitogen used for EAE induction. In clinical trials, Cop 1 was found to slow the progression of disability and reduce relapse rate in exacerbating-remitting MS patients. As an antigen-specific intervention, treatment by Cop 1 has the advantage of being less likely to cause long-term damage.

The mechanism of Cop 1 activity in EAE and MS seems to involve, as an initial step, the binding of Cop 1 to major histocompatibility complex (MHC) class II molecules, thus competing with myelin antigens for T-cell activation, both at the MHC and T-cell receptor level. Cop 1 also induces specific suppressor cells of the Th2 type, which probably play a major role in its mechanism of action. Th2 cells cross the blood-brain barrier and accumulate in the CNS, where they can be stimulated in situ by MBP and thereby exert their therapeutic effects in the diseased organ. This therapeutic effect was manifested, in the brains of EAE-induced mice, by a decrease in inflammatory cytokine IFN-γ and by secretion of the anti-inflammatory Th2 cytokine IL-10 in response to the autoantigen MBP. More recent clinical results show that after six years of treatment, 152 of 208 (73%) of the patients who entered the open-label trial continue to feel that they are benefiting from continuing treatment. The high patients compliance is backed up by a major decrease in relapse rate (72%) accompanied by the fact that the majority of patients has not deteriorated during the six years.

Treatment with Cop 1 (parenteral as well as oral) induces specific Th2 cells in the central nervous system (CNS), where the pathological processes of EAE and MS occur. This was manifested by isolation of Cop 1 specific Th2 cells from brains and spinal cords of actively sensitized mice, as well as by their localization in the brain, after their passive transfer to the periphery. Cop 1 treatment results in the accumulation in the CNS of cells that secrete Th2 cytokines in response to either Cop 1 or MBP, even in Th1 shifting environment, which consequently leads to a therapeutic effect in the MS diseased organ.

Whereas MS is mainly a T-cell-mediated disease, myasthenia gravis is a T-cell-regulated disease mediated by antibodies against the acetylcholine nicotinic receptor (AChR). Nevertheless, T-cells are of crucial importance for the formation of these antibodies. In our study, analogs have been synthesized of two immunodominant myasthenogenic T-cell epitopes (p195-212 and p259-271) derived from an a-subunit of the nicotinic AChR. Ideally, the goal of therapy for MG should be the elimination of autoimmune responses to the AChR specifically, without interfering with immune responses to other antigens. To this end, the dual analog composed of the tandemly, reciprocally arranged two single analogs of p195-212 and p259-271, namely Lys262-Ala207, was prepared and shown to efficiently inhibit the proliferation of T-cell-lines specific to the myasthenogenic peptides, and of lymph node cells primed in vivo to either of these peptides The dual analog specifically inhibited in vitro T-cell stimulation to either myasthenogenic peptide in >90% of the responding MG patients. When administered orally, the dual analog could treat EAMG induced in mice by immunization with the multi-determinant native Torpedo AChR. Moreover, it had beneficial effects on the clinical manifestations characterizing EAMG.

Thus, the dual analog is an efficient immunomodulator of EAMG in mice and could be of specific therapeutic potential for MG. The dual analog vaccine candidate acts by specifically and actively suppressing myasthenogenic T-cell responses. This active suppression is mediated by the upregulation of transforming growth factor-b (TGF-b) secretion and downregulation of IFN-g and IL-2 (Th1 type cytokines). A state of non-responsiveness is induced by the dual analog, which, at least partially, causes the cells to undergo anergy. Thus, the dual analog can definitely be considered as a candidate for a therapeutic vaccine.

Diverse Pathways to Achieve regulation of Autoimmune Responses

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Because of the importance of regulation in the prevention of autoimmunity, there are many redundant devices that are used by individuals to control autoreactivity. We would like to describe three diverse levels at which this is accomplished. First, in the disease model for MS, (experimental autoimmune encephalomyelitis=EAE) the B10.PL mouse manages to control its disease very efficiently by employing a TcR-centered regulatory circuit. This feedback system in the B10.PL mouse manages to control the paralytic response to an antigen such as myelin basic protein , by shutting down the response after its inception. We have discovered that this comes about through the combined effort of a CD4 and a CD8 regulatory cell, each of which recognizes a different antigenic determinant on the Vb8.2 TcR. The activated CD4 T cell, specific for MBP then undergoes apoptosis owing to the killing effects of the CD8 T cell, achieving a longterm cure of the mouse, and resistance to further attempts to induce autoimmunity to the same antigenic system. A second example occurs in the NOD mouse in which T cells specific for a determinant on GAD, glutamic acid decarboxylase, are able to down-regulate the spontaneous diabetes that arises in this strain by adoptive transfer of specific CD4 regulatory T cells. In transwell experiments, it was shown that the active agent was IL-13: thus, this mechanism of regulation involves Th2-Th1 cytokine interactions. The final mechanism of regulation occurs at the MHC level, where two different MHC molecules compete for binding of several determinants along a multideterminant strand of the antigen. If one of the better binding MHC molecules is able to capture one of the determinants along this multideterminant strand, it can potentially prevent a dangerous T cell from becoming activated. These types of mechanisms can occur concomitantly in any single situation, and provide a diverse set of alternatives to CD4CD25 T cell regulation.

Dominant Tolerance in Anti-tumor Autoimmunity

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The potential influence of regulatory mechanisms on tumor biology and immunotherapy has not been extensively explored. Active immunization to Epidermal Growth Factor (EGF) has been studied both in experimental animals and in human cancer patients. The ultimate goal has been to exploit autoantibodies as a way to remove or inactivate self-EGF, aimed at reducing the growth rate of EGF-dependant tumors.

We therefore evaluated the contribution of peripheral regulatory mechanisms into autoimmunity based on deliberate immunization to this autologous growth factor; and the degree to which regulatory T-cells impede tumor immunity. The effect of transient depletion of CD4, CD8 and CD25 T cells in the induction phase of the immunoresponse against EGF in BALB/c and C57BL/6J mice was studied. We found that T cell depletion enhances the immunoresponse against the EGF in a dose and schedule dependent manner. The specific anti-EGF autoimmune response enhancement correlates with the antitumor response to the highly-metastatic Lewis lung carcinoma (3LL-D122) model.

We conclude that a precise description of regulatory T-cells population behavior in cancer patients could lead to a rational combination of self-antigen cancer vaccines applied in circumstances where immunoregulation may be brought under temporary control so as to improve antitumor immunity.

Listeria Monocytogenes as a Live Bacterial Vector for Tumor Antigens: Can It Teach Us What Is Required for Effective Tumor Immunotherapy?

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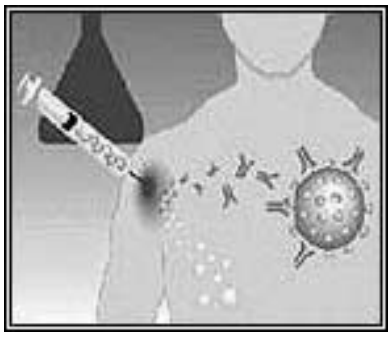
HPV E6 and E7 antigens have been an intense focus of cancer immunotherapies using a variety of vaccine vectors. Because of the intra-cellular localization of these antigens, these therapies are mostly directed at inducing cellular immune responses of the type that is the forte of L. monocytogenes. We engineered expression systems for HPV-16 E7 in L. monocytogenes. In one the bacterium secretes a fusion protein composed of a truncated, non-hemolytic LLO, joined at the C-terminus to E7. In a second recombinant, Lm-E7, the E7 protein is secreted using only the signal sequence of hemolysin. When we use these vectors to treat mice bearing established HPV-16 immortalized tumors we find that Lm-LLO-E7, induces complete tumor regression in 70% of syngeneic mice, whereas Lm-E7 has very little impact on tumor growth. This surprising result does not correlate with the ability of these vectors to induce CD8+ T cell reponses, rather it correlates with the ability of T cells to home to and penetrate the tumor site. A further question we have answered is why delivery of E7 as a fusion protein with LLO results in improved anti-tumor efficacy compared to the use of E7 alone. In Lm-LLO-E7, the fusion to LLO may enhance the immunogenicity of the antigen because of the presence of a PEST domain within LLO. PEST domains are thought to target proteins for ubiquitination and degradation by the proteasome that may lead to increased antigen presentation and enhanced immune responses. To confirm the role of the PEST sequence we designed intermediates between the two constructs. A version of Lm-LLO-E7 in which the PEST sequence was excised was not effective in inducing tumor regression. In contrast, Lm-PEST-E7, which contains only the PEST domain of LLO fused to E7, was as effective as Lm-LLO-E7. In summary, our studies indicate that fusing this PEST domain to E7 increases the effectiveness of the anti-tumor response in a mouse tumor model. We also show that induction of CD8+ T cells may be a necessary but not a sufficient condition for good anti-tumor efficacy.



Immunotherapy for the New Century

Inmunoterapia en el Nuevo Siglo

December/Diciembre 5-8, 2002
La Habana, Cuba

Session: Cellular and Molecular Bases of Peripheral Tolerance

T Cell Tolerance to Gastric Autoantigens and the Role of CD4+ CD25+ Regulatory T cells

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Experimental mouse autoimmune gastritis has been established as a highly defined model of organ-specific autoimmunity. Autoimmune gastritis represents one of the few autoimmune diseases in which the causative autoantigens, namely the gastric H/K ATPase a and b-subunits, are defined. Furthermore, it has been clearly established that a CD4+ T cell response to the H/K ATPase b-subunit, in particular, is essential for the initiation of autoimmune gastritis. The immunopathology of autoimmune gastritis is due to a disruption of the normal developmental pathways of the mucosa, rather than depletion of the end-stage gastric parietal and zymogenic cells. CD4+ CD25+ regulatory T cells were first described in experimental autoimmune gastritis and there is now considerable interest in the potential role of these immunoregulatory T cells in protection against a variety of autoimmune diseases. In addition, the availability of H/K ATPase deficient mice has began to provide considerable insight into the basis for tolerance to the gastric autoantigens. This presentation will focus on (1) the mechanisms that underscore T cell tolerance to the gastric H/K ATPase b-subunit (H/Kb), (2) the specificity of CD4+ CD25+ regulatory T cells that protect against autoimmune gastritis in experimental models and (3) the role of CD4+ CD25+ regulatory T cells in normal individuals.

Firstly, we have explored the extent to which endogenous H/Kb contributes towards tolerance of the H/Kb-specific T cell repertoire in normal individuals. By comparison of T cell responses in H/Kb-deficient (o/o) and H/Kb-expressing BALB/c mice, we have shown that the endogenous H/Kb autoantigen plays a major role in the tolerance of pathogenic H/Kb-specific T cells. Experiments will be presented which demonstrate that the H/Kb specific T cells in wild-type mice represent the residue of a highly pathogenic T cell repertoire that has been subjected to partial tolerance induction. Secondly, using H/K ATPase deficient mice, and transgenic mice we have generated which lack a spectrum of other gastric antigens, experiments are in progress to define the antigen specificity of the gastritis protecting CD4+CD25+ regulatory T cells. Thirdly, we have examined the role of the CD4+ CD25+ regulatory T cells within the normal individual by assessing the affect of depletion of CD4+ CD25+ regulatory T cells in mice on the induction of organ-specific autoimmunity. Our data suggests that CD4+ CD25+ regulatory T cells may be important in protection against autoimmunity while the immune system is being established in young animals, but subsequently other factors are required to initiate autoimmunity.

Modelling the Interactions Between Regulatory and Effectors T Lymphocytes

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We have designed four models representing the population dynamics of regulatory and target T cells, which implement alternative mechanisms of linked suppression previously proposed in the literature. We made phase plane and bifurcation analysis of each model, and identified its pros and cons in terms of the relationship with the large body of in vivo experimental observations on T cell mediated tolerance. We argued that accounting for the quantitative details of adoptive transfers of tolerance requires models that: (i) possess bitable regimes in which either regulatory or target T cells dominate the steady state interpreted as tolerance and immunity respectively, depending on initial conditions; and (ii) possess a steady state interpreted as tolerance in which both regulatory and target cell populations coexist. We showed that only models in which the growth of regulatory T cells is strictly dependent on their target T cells bear these two properties. To further assess these candidate models, we challenged them by their capacity to explain observations in vitro. This analysis allowed us to quantify the efficiency of in vitro suppression dependent on multicellular conjugates and to show that it has an upper bound. Comparing this upper bound with the efficiency of suppression in vitro, we rejected those models in which suppression is mediated by simple competition for APCs or in which the regulatory T cell population is unable to grow. Thus, as a whole our theoretical analysis of alternative models allowed to narrow down the number of candidate hypotheses to those in which the population regulatory T cells grows as a function of the target T cells they suppress. The growth of this population may be driven by a target cell-dependent growth factor and/or may result from differentiation of target cells to the regulatory phenotype. We addressed and confirmed the first hypothesis by designing and carrying out new in vitro suppression assays in which proliferation of regulatory CD4+CD25+ and target CD4+CD25- T cells could be quantified concomitantly. This study showed for the first time that regulatory CD25+ T cells, which are unable to proliferate by themselves when stimulated in vitro, will proliferate when costimulated together with target CD25- T cells. Moreover, the proliferation of regulatory cells is correlated with the expansion of the target cell population suggesting that the two cell types might share a target cell-dependent growth factor, whose production is inhibited by regulatory T cells. The nature of this growth factor is discussed to be most likely IL-2 and it is argued that this would provide a mechanistic rational for the involvement of IL-2, IL-2 receptor and CD4+CD25+ T cells in self tolerance.

Immune Dysfunctions Caused by Myeloid Suppressor Cells in Tumor-Bearing Mice

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The failure of the active immunotherapy of cancer does not exclusively depend on the incapability to prime antitumor lymphocytes. Indeed, in tumor-bearing mice the ability of the immune system to detect and destroy tumor cells can be deeply compromised. Several studies suggest that this suppression is not maintained by a failure of the immune effector cells but is rather imposed by a peculiar set of suppressor cells elicited by tumors. T lymphocyte inhibition by myeloid suppressor cells (MSC) was clearly documented in mice during tumor growth and elimination of suppressor cells, either in vitro or in vivo, completely reversed the dysfunctional T cell responses. Also immunization with powerful vaccines encoding tumor antigens or the use of cytokines, such as IL-2 or GM-CSF, can cause the appearance of MSC, thus limiting the therapeutic efficacy of these treatments. In mice, suppressor cells represent a heterogeneous population comprising immature myeloid cells ($CD31^+/CD11b^+/Gr\text{-}1^+$), monocytes, and granulocytes that inhibit T lymphocytes by triggering their apoptosis. Characterization of the immunosuppressive pathways used by MSC is noteworthy because it may lead to strategies for reversing tumor-induced dysfunctions that support the development of effective anti-tumor immunity.

Analysis of Antibody Reactivity Patterns by Quantitative Immunoblot

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If "natural antibodies" resulted just from immune responses to arbitrarily encountered antigens, robust reactivity patterns would not be expected. But such patterns exist, emerge and develop spontaneously in normal organisms, and remain during life, but show characteristic changes in autoimmune diseases. This is interpretable as a reflection of stable systemic states, actively maintained by the immune system. Antibody reactivity patterns can be systematically and reproducibly obtained by a standardized quantitative immunoblot technique ("Panama-Blot"), and evaluated by multivariate analysis. More recently, this method was employed to describe immunoreactivity patterns in human patients affected with parasitic diseases in studies conducted by our group. IgG reactivity profiles to human brain proteins, and even to an arbitrarily chosen E. Coli extract, showed clear differences between human malaria patients, asymptomatic parasite carriers and unexposed individuals. Equally, clinical forms of human Schistosoma mansoni parasitosis could be differentiated. In summary, the analysis of antibody reactivity patterns with the aim of describing systemic regularities, thus systemic states, appears as an interesting approach towards immune activity that is applicable in various contexts - also including the possibility of monitoring specific immunotherapy.

Exploring T Cell Specificity with Combinatorial Peptide Libraries

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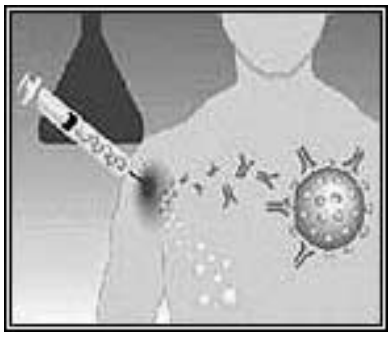
Very large synthetic combinatorial libraries can be used to identify extensive arrays of peptide ligands specific for T cell clones and lines of clinical relevance for further analysis in various models of immune regulation and the design of vaccines for treatment of infectious and autoimmune diseases and cancer. These peptide arrays defined for a single T cell line or clone include superagonists with activities several orders of magnitude greater than the native peptide sequence (when known), partial agonists and antagonists which provide a basis for analysis of TCR/peptide/MHC molecular interactions that lead to different states of T cell activation and antigen induced cell death. In addition to providing information on the sequence motifs most appropriate for MHC binding, they also indicate sequence motifs most effective as TCR contact residues for stimulating T cell lines of a given specificity. As a consequence, this approach is especially useful for defining optimized peptide sequences most effective in up-regulating T cell mediated immune responses against weak antigen systems such as those frequently encountered in anti-tumor immune responses, and may be useful in the design of down-regulatory "suicide" sequences that destroy pathogenic T cells in autoimmune disease.



Immunotherapy for the New Century

Inmunoterapia en el Nuevo Siglo

December/Diciembre 5-8, 2002
La Habana, Cuba

Session: Tolerance and Autoimmunity (continued)

Immunization with Plasmid DNA Expressing Islet (Auto) Antigens and IL-4 or IL-10 Enhances Protection from Type 1 Diabetes

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Previous investigations have shown that immunization of prediabetic NOD or RIP-LCMV mice with plasmid DNA expressing insulin B chain or glutamic acid decarboxylase (GAD) can prevent disease development in approximately 50% of the animals (1-7). This therapeutic success is associated with a modulation of the cytokine profile of islet antigen specific T cell responses. Increased production of IL-4 and IL-10 is noted and, in RIP-LCMV mice, protection can be transferred by insulin-specific CD4+ regulatory lymphocytes. These cells produce high levels of IL-4 and IL-10 and act as bystander suppressors of autoaggressive CD8 lymphocytes in the pancreatic draining lymph node likely through modulating local antigen presenting cells.

Our goal was to increase the efficacy of this systemic antigen-specific immune modulation by adding plasmids expressing IL-4 and/or IL-10 at the time of immunization. Indeed, prevention of diabetes using insulin-expressing plasmids was enhanced to 85% when combined with IL-10. This was associated with a complete lack of insulin-specific autoantibodies. Interestingly, there was a firm trend between lower levels of autoantibodies and protection from diabetes by DNA vaccination. This may possibly be of value for future trial endpoint design. On the T cell level, addition of IL-10 resulted in a reduction of insulin specific interferon-g producing CD4+ lymphocytes. No augmentation of aggressive CD8 responses was noted following these vaccines.

In summary, we would propose the use of similar DNA vaccinations to prevent human autoimmune diseases. The concern of induction or augmentation of autoaggressive responses can be overcome by adding biologically active response modifiers, (8) such as IL-4 or IL-10, at the time of immunization, which appears to enhance the degree of protection. This is of particular value, since IL-10 has been implicated as an important regulator of APC function in mice and humans, is produced by regulatory ('TR1') lymphocytes, and was recently found elevated in diabetic individuals who received anti-CD3 treatment to prevent worsening of disease (9). Regular assessment of islet-antibodies could function as a trial correlate and predict the degree of success sufficiently early. It is a possibility that combination of antigen specific immunotherapy with non-antigen specific approaches, such as systemic anti-CD3 administration, may be important for achieving long-term protection, while avoiding systemic side effects.

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2. Plasmid vaccination with insulin B chain prevents autoimmune diabetes in non obese diabetic mice. Bot A, Smith D, Bot S, Hughes A, Wolfe T, Wang L, Woods C, von Herrath M. J Immunol 2001 Sep 1;167(5):2950-5
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8. Endogenous expression levels of autoantigens influence success or failure of DNA immunizations to prevent type 1 diabetes: addition of IL-4 increases safety. Wolfe T, Bot A, Hughes A, Mohrle U, Rodrigo E, Jaume JC, Baekkeskov S, von Herrath M. Eur J Immunol 2002 Jan;32(1):113-21
9. Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 2002 May 30;346(22):1692-8

Using Regulatory APCS to Induce/Mantain Tolerance

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Regulation of auto-aggressive immune responses can occur locally in the target organ or its draining lymph node and is a principal goal of many novel "immunotherapeutic" strategies devised to prevent or treat autoimmune diseases such as type 1 diabetes or multiple sclerosis. These type of interventions are based on the more recent hypothesis that each inflammatory auto- immune process is composed of auto-aggressive and autoreactive regulatory responses that are in a delicate balance. The goal of this work is to determine the conditions that lead to the generation of regulatory APCs, and to dissect the precise mechanisms through which they downregulate auto-aggressive lymphocytes resulting in reduction or non-progression of islet infiltration and prevention of type 1 diabetes. We have found evidence that exposure of APCs to IL-4 or IL-10 leads to their inability to further propagate or potentially down-modulate auto-aggressive T cell responses in vitro. The conditions that lead to the generation of regulatory APCs will be further investigated by in vitro culture of APCs with various cytokine treatments, followed by transfer into prediabetic RIP-LCMV recipients. Findings relating to the efficacy of treatment with regulatory APCS will be discussed, as well as their phenotype, and the effect they have on auto-aggressive lymphocytes *in vitro* and *in vivo.* These studies should help us understand the central coordinating role of APCs in autoimmune processes and employ them therapeutically.

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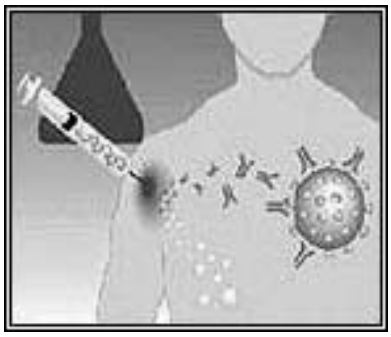

Immunotherapy for the New Century

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December/Diciembre 5-8, 2002
La Habana, Cuba

Session: Regulatory Functions of Antibodies

Superantibodies —The Next Generation

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The structure of immunoglobulins is divided into two main domains according to the duality of biological functions: the targeting to antigen and the biological effector functions that activate various defense mechanisms. Beside of this orthodox architecture a rare type of special antibodies has been discovered over the years. These naturally occurring antibodies add to the biological duality a third activity, such as homophilic dimerization, membrane penetration and catalytic function. This class of antibodies has been termed "Superantibodies" [1]. We have recently developed a method to create superantibodies that mimic the rare natural Superantibodies.

Our approach is to transplant biologically active peptides mediating a desired activity to antibodies using an affinity photocrosslinking technology. For example, we generated homodimering antibodies with increased targeting and induction of apoptosis in tumor cells. Other man-made superantibodies are antibodies that penetrate the cell or nuclear membrane without harming the living cell.

The universality of the technology allows to generate therapeutic and diagnostic antibodies with improved utility and therapeutic value.

1. Immunol Today 1998;19:221-7.

Phage Display Engineering of Magic Bullets for Induction of In Vivo *V_h Targeted Apoptotic Deletion of B-Cells in Mice and Non-Human Primates*

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We have performed studies to characterize the mechanisms by which protein A of Staphylococcus aureus (SpA) induces death of VH targeted B lymphocytes. From recent crystallographic analysis, we have recently shown that these activities derive from the binding capacity of SpA for conserved variable region framework subdomains of the antigen receptors of lymphocytes rather than the CDR involved in binding conventional antigens. These interactions enable SpA to target V_H regions encoded by clan V_HIII genes, which have been conserved in the B-cell repertoires of mammalian species. To define the mechanisms responsible for B-cell effects, we administered SpA into genetically modified and Ig transgene expressing mice, and then followed the fate of affected B cells. Within hours of exposure, a sequence of events was initiated in SpA-binding splenic B cells, with rapid down-regulation of sIgM, CD19 and CD21, and the induction of an activation phenotype. Apoptosis followed through a process heralded by dissipation of mitochondrial membrane potential, the induction of the caspase pathway and DNA fragmentation. While apoptosis did not require the Fas death receptor, B cells were protected by overexpression of Bcl-2 or by stimulation with IL-4 or CD40L. These studies indicate that the oligovalent structure of SpA, which enables high affinity V_H targeted BcR cross-linking, induces apoptotic cell death, resulting in immunomodulation of B-cell responses. To evaluate the relevance of these activities to the primate immune system, intravenous infusions were performed in the old world monkey, Macacus fascicularis (Cynomologus macaque), in which 15-30% of peripheral blood B cells express V_H3 family genes that conveys the capacity for Fab-mediated SpA binding. Within two days of SpA infusion, flow cytometric studies demonstrated the specific induction of intracellular activated Caspases and DNA fragmentation in peripheral V_H3-B cells, documenting the induction V_H targeted apoptotic death. In recent studies we have used phage-display methods to generate libraries of variant SpA-domain libraries degenerate at 6 positions implicated in Fab-mediated binding of B cells. Following selection of libraries with these domains displayed on filamentous phage, we have isolated variant domains with unnatural amino acid sequences, that demonstrate enhanced in vitro binding characteristics, and the in vivo biologic properties are now being studied. These findings advance the development of B-cell superantigen based therapies that may prove useful for the targeted deletion of pathogenic B cells that contribute to certain autoimmune diseases

Immunotherapy of B-cell Tumors

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Idiotypic determinants of the immunoglobulin of malignant B cells serve as tumor specific antigens. Our studies focus on two approaches to target idiotypes (Id) for immunotherapy:

Induction of Id-specific anti-tumor immune responses by immunization with autologous immunoglobulin. The effector mechanisms, which destroy B-cell tumors following Id-vaccination, are a controversial issue. Id-specific T cells have not been well studied, mainly because their low frequency does not allow detection by traditional methods. We applied the highly sensitive ELISPOT assay for enumeration of Id-reactive T cells in murine models of Id vaccination. We demonstrated that immunization induced a significant increase in the frequency of Id-specific IFN-ã-secreting T cells and that both CD4 and CD8 T cells are involved in the response to Id. Whereas B-lymphocyte tumors can be rejected by anti-Id antibodies, cell-mediated immune responses play a predominant role in destruction of plasma cell tumors. We demonstrated that protection against plasma cell tumors in Id-vaccinated mice is mediated by several mechanisms, part of which depend on Id secretion by the malignant cells, while others do not depend on immunoglobulin secretion.

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Idiotypic determinants of the immunoglobulin of malignant B cells serve as tumor specific antigens. Our studies focus on two approaches to target idiotypes (Id) for immunotherapy:

Application of bispecific antibodies, directed against Id and against adhesion molecules, to inhibit dissemination of B-cell lymphoma. Antibodies to adhesion molecules can block tumor metastasis. However, they may also block normal functions of non-malignant cells. To circumvent this adverse effect, we proposed the use of bispecific antibodies that bind simultaneously to an adhesion receptor and to a tumor specific antigen of an individual cell. Such antibodies bind more avidly to tumor cells that coexpress both target antigens, than to normal cells that express only the adhesion receptor. We therefore produced a bispecific antibody with specificity to the adhesion molecule LFA-1 (CD11a/CD18) and to the Id of a murine B-cell lymphoma. We demonstrated that this antibody blocks lymph node and liver metastasis in mice carrying subcutaneous tumors. In contrast to anti-LFA-1 antibodies that block immune responses, such as delayed-type hypersensitivity, the anti-Id x anti-LFA-1 bispecific antibody has no effect on these immune responses. Hence, bispecific antibodies against adhesion molecules and against tumor specific antigens may selectively block tumor metastasis in a way, which may leave much of the normal tissue intact.

Immune Responses in Breast Cancer Patients Immunized with an Anti-idiotype Antibody Mimicking NeuGc-containing Ganglioside

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A phase I clinical trial was conducted in patients with stage III/IV breast carcinoma who were treated with the anti-idiotype mAb 1E10 specific to an Ab1 mAb able to react specifically with N-glycolyl-containing gangliosides and with antigens expressed on human melanoma and breast carcinoma cells. Patients were treated with 1 or 2 mg of aluminum hydroxide-precipitated 1E10 mAb, every other week for six injections. Two patients of each dose were reimmunized 7-9 months after finished the induction phase. There were not differences between the two levels of dose tested in relation to toxicity and immunogenicity. No evidences of serious or unexpected effects were observed. In hyperimmune sera from eight of the nine patients who received at least four doses of anti-Id vaccine preparations, strong specific responses were observed both against 1E10 mAb and NeuGc-GM_3 ganglioside (Ab3 Id+Ag+). These results showed an "internal image" behavior for 1E10 Ab2 mAb in humans, in contrast with our previous results obtained in mice, rabbit and monkeys. Strikingly, Ab1' antibodies able to bind to NeuGc-containing gangliosides, but not to 1E10 mAb (Id-Ag+) were detected in immunized patients' sera.

Monitoring HLA Class I Antigen-tumor Antigen Peptide Complexes

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HLA class I antigens play a major role in the interaction of malignant cells with host's immune system, since they present tumor antigen (TA) derived peptides to cytotoxic T lymphocytes (CTL). To date, a number of studies have clearly demonstrated that HLA class I antigens as well as components of the antigen processing machinery (APM) are frequently downregulated and/or lost in malignant lesions. These abnormalities are significantly associated with a poor prognosis and can have a detrimental impact on the outcome of T cell-based immunotherapy. However, previous investigations have primarily focused on the analysis of the expression of HLA class I subunits, TA and/or select components of APM in malignant lesions. The disadvantage to these studies is that they do not give any indication of the actual level of HLA class I antigen-TA peptide complex expression. Furthermore, these studies do not provide information about the function of the proteins expressed, as well as, the functional implications of downregulation of components of the APM.

Recent studies have demonstrated the isolation of antibodies that recognize the HLA-A*0101-$MAGE1_{161-169}$ peptide complex via direct selection from a phage display antibody library. The goal of this work was to investigate the use of a semi-synthetic phage display antibody library (scFv) as a source of probes recognizing the HLA-A2*0201-$MART1_{27-35}$ peptide complex. We have chosen HLA-A2*0201-$MART1_{27-35}$ because of the high frequency of its expression in melanoma, its use as a target in T cell-based immunotherapy and the high immunogenicity of MART1 observed in melanoma patients. Three rounds of selection were performed on recombinant HLA-A2*0201-$MART1_{27-35}$ derived peptide complexes and resulted in the isolation of $9x10^7$ clones. Three unique scFv fragments (MA1, MA2 and MA3) were isolated that were capable of specifically recognizing the HLA-A*0201-$MART1_{27-35}$ derived peptide complex on the surface of $MART1_{27-35}$ pulsed human ly mphoid T2 cells as well as HLA-A*0201(+), MART1(+) human melanoma cell lines. These clones did not demonstrate any reactivity with $gp100_{161-169}$ pulsed human lymphoid T2 cells as well as HLA-A*0201(-), MART1(+) and HLA-A*0201(+), MART1(-) melanoma cell lines. The development of these probes will allow one to directly monitor HLA-A2*0201-$MART1_{27-35}$ peptide complex expression in malignant lesions and assess the clinical significance its expression in malignant disease.