Mining at the Intersection Between Cancer Research and Autoimmunity Research

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REPORTES

It is difficult to summarize the context in which current immunotherapy research is done, and this is because there is no "one context" but two: a practical context and a theoretical context; and they do not overlap each other too much.

The practical context is mainly given by the products for cancer immunotherapy under clinical trials: more than 100 products, mainly monoclonal antibodies and cancer vaccines, all surrounded by an atmosphere of enthusiasm. Eight monoclonal antibodies have already entered the market (three of them for cancer) and there are more than 20 in advanced clinical trials. Nobody doubts anymore that monoclonal antibodies will be in our pharmacopoeias. The polemics is now shifted to whether they will be chimeric, humanized or fully human antibodies, and to whether they will be manufactured in fermenters, or in transgenic plants or animals. Scientific literature about cancer vaccines, which was hard to find just 10 years ago, is growing exponentially (Figure 1).

But it happens that basic science usually moves faster than clinical research, and during the long time while monoclonal antibodies and vaccines were going through Phase I-II-III clinical trials and regulatory compliance, the picture of Fundamental Immunology was changing and moving in a different direction.

What essentially happened in immunological thinking in the last decade of the 20th century is that it started to move away from the clonal selection theory and from the idea of deletional tolerance.

Here is a summary of what the 90's decade left behind:

1. The critics to clonal selection theory
2. Dominant tolerance
   - the evidences of physiological recognition [1]
   - the phenomenology of "dominant tolerance" and the initial characterization of regulatory T cells [2]
   - the experiments about "linked suppression" [3]
3. The essential role of antigen presentation
   - the molecular basis of dominance/cripticity and determinant spreading [4]
   - the physiology of the antigen presenting cells (APC) (dendritic cells) [5]
   - context-dependent immunity and the "danger" theory [6]
   - the links between innate immunity and adaptive immunity (a reinterpretation of the second signal) [7]
   - glycolipid presentation to T lymphocytes [8]
4. The Th1/Th2 (Th3?) choices: a repertoire of effector responses [9]
5. The measurements of lymphocyte population dynamics [10]
6. IL-12 to IL-18

This picture is different from the theoretical background on which the antibodies and the vaccines that are being tested today were based.

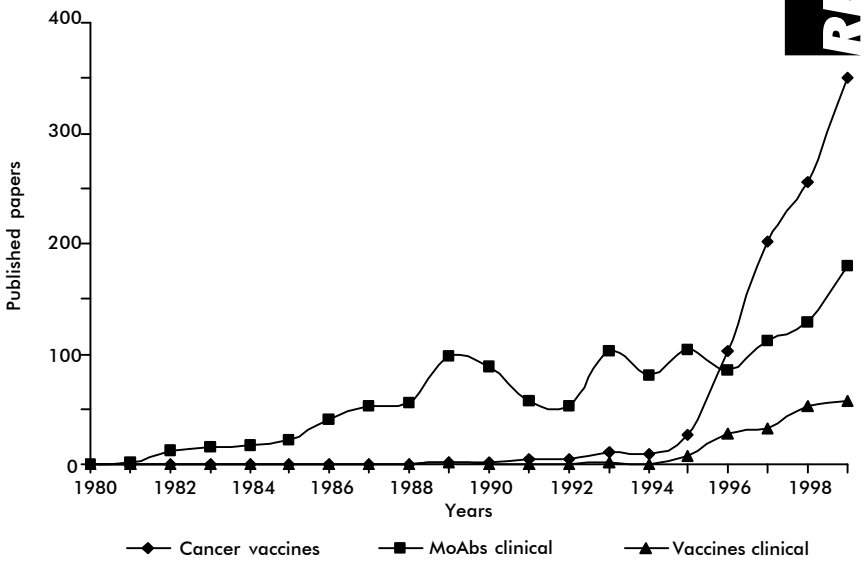

Figure 1. Scientific literature published about cancer vaccines, 1980–1998.

This is the gap and this Meeting was about filling it.

In fact, dominant tolerance, the "danger" theory and the Th1/Th2 dichotomy are providing paradigms alternative to clonal selection for explaining the choices between ignorance, tolerance and aggression that the immune system is constantly doing.

The story started with the increasing description of natural autoantibodies [1], and later on the physiological existence of T cells specific for self-epitopes [11]. Later on, it was surprisingly found that the antigens that patient's lymphocytes recognized in tumors were more often normal self-proteins than mutated proteins [12].

If many self-antigens are normally seen by circulating T and B lymphocytes, and tolerance to these antigens cannot be explained by clonal deletion, then, how is tolerance achieved?

Alternative explanations came through two directions

The first is "dominant tolerance", an idea that was inspired by the observation that lymphopenia (either congenital or induced) is associated with autoimmune diseases [13]. During the last ten years, more controlled experiments were done showing that autoimmunity can be produced if animals are depleted of an specific subset of CD4- T lymphocytes (CD25+), and that a small amount of these "regulatory" lymphocytes is enough to control potentially auto-aggressive T cells [2].

The picture was completed by experiments showing two features of dominant tolerance: Firstly it behaves infectiously [14], which means that the contact of regulatory T cells with naïve T cells can "teach" tolerance

Summary of the Opening Lecture at the Meeting: "Immunotherapy for the New Century", Havana, Cuba, November-2000.

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to the naïve cells and make them become in turn regulatory themselves; and secondly it exhibits "linked suppression" [3], which means that if a new antigen is presented in the same antigen-presenting cell together (linked) with a previously tolerated antigen, the new antigen will be tolerated.

As we will see below, these findings have a tremendous importance for the design of cancer vaccination procedures.

The second alternative explanation of tolerance was nurtured by the accumulation of knowledge about antigen presentation and the physiology of dendritic cells. It was found that, before becoming fully competent to activate T lymphocytes, dendritic cells need to undergo a process of maturation in which they increase the expression of major histocompatibility complex (MHC) and co-stimulatory molecules. It was also found that this maturation was induced by bacterial products, lipopolysaccharides, glycans, manans, bacterial DNA, TNF, IL-1; i.e., the molecules of the innate immune system [5].

These findings identified a link between innate immunity and adaptive immunity, giving the first a kind of "instructive role" [6] to indicate to the rest of the system whether to react or not.

Additional support to this idea came from the finding that coupling of a complement molecule (C3d) to antigen dramatically reduces the amount of antigen required for a response [15], and from the identification in human cells of a molecule analogue to the *Drosophila* toll protein, which mediates anti-infectious defense in the fly and potentiates T-lymphocyte response in human cells [16].

These results were interpreted in terms of the so called "danger theory" which states that the immune system does not differentiate between what is self or nonself, but between what is dangerous or innocuous [6].

This theory has provoked a lot of polemic, but whatever theoretical construction is done what seems now evident is the decisive importance of the "context" in which an antigen is first seen by the immune system.

The antigen presentation field has been further complicated by the identification of different sub-sets of dendritic cells and different functional properties, probably associated with the capacity of directing T-helper response either towards Th1 or Th2 phenotypes [17], by the discovery of the effects of T lymphocytes on dendritic cells [18], and by the discovery that non-peptidic molecules can be presented to T lymphocytes not restricted by the MHC, but by CD1 molecules [8].

For a more complete description of the new theoretical context built during the 90's decade, we shall also look at the amount of new data about lymphocyte population biology [10] describing the independent homeostatic regulation of T and B lymphocytes, and of resting and memory lymphocytes; identifying the requirement of T-cell receptor engagement for naïve T-cell survival (but not for memory T cells); and discovering the recursive selection of diverse immune repertoires in periphery even in the absence of foreign antigens. Lymphocyte population biology (unlike dominant tolerance) still lacks a proposal of a unifying theory, but it will probably appear soon.

Are these recent achievements in Fundamental Immunology making an impact in clinical cancer immunotherapy? Obviously not. We should ask ourselves why.

Since the 1996 edition of the present Immunotherapy Meetings in Havana, we have been discussing that the classic approach of cancer immunotherapy—looking for neoantigens, dominant epitopes and unspecific immunostimulation—was probably an oversimplification of reality. It was trying to reproduce—for cancer treatment—the way the immune system reacts to a foreign pathogenic microbe.

Cancer immunotherapy in the new century will probably look more like the induction of a controlled autoimmune disease: self–antigens, cryptic epitopes, immunosuppression procedures [19, 20].

Cancer immunologists have a lot to learn from autoimmunity research: this is the "mining" concept

"Mining" makes sense when resources are available and the extraction–processing capacity is the limiting factor. This is exactly the current situation in cancer immunology: there are abundant knowledge resources created by basic immunology and autoimmunity research, which could be exploited if we are able to extract and process them.

To extract and process knowledge from basic science means building links and requires more research. The identification of pieces of new research that are required to move basic science to the clinical setting is not trivial.

A starting list of these "missing links" could be the following:

1. A systematic description of antigens/epitopes of cancer cells recognized by patient's T/B lymphocytes,
2. An operational definition and a description of the "dominant self",
3. A systematic study of immune response kinetics during immunization with self-antigens,
4. Direct comparisons of immunizations with dominant and "cryptic self" epitopes,
5. More data about the phenotype and population dynamics for regulatory T cells,
6. A systematic evaluation of the effect of diverse immunosuppressors on regulatory T cells,
7. A systematic evaluation of diverse protocols combining vaccination and immunosuppression procedures,
8. To complete the inventory of ligands of innate immunity receptors,
9. The identification of structural regularities in the ligands of innate immune receptors (bio-informatics approach),
10. A systematic evaluation of innate immunity molecules in composition the vaccines.
11. A meta-analysis of dendritic cell vaccines,
12. The identification of regulatory idiotopes on T-cell receptors and antibodies,
13. A re-assessment of tumor escape mechanisms (second wave).

Dominant tolerance, innate immunity, the danger theory, T-helper cell diverse phenotypes, and lymphocyte population biology (the cognitive gifts of the 90's decade) are creating huge possibilities to be explored in cancer immunotherapy.

Nobody can afford doing all the required research. The issue is to focus smartly and mine where the gold is.

Let this meeting help to draw the map.

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Tumor Immunology and Cancer Vaccines

T-cell Recognition of Glycolipids: Molecular Requirements for Immunogenicity

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We have identified and characterized human T-cell clones which recognize self-glycolipids such as GM1 ganglioside, sulfatide and galactosylceramide, in association with CD1 antigen-presenting molecules. These unusual T cells are more abundant in patients with Multiple Sclerosis, Guillain-Barré syndrome and with bacterial infections. This new type of antigen specificity suggests that glycolipids may be new candidate molecules to activate T cells in modern immunotherapy. The molecular requirements for CD1 association and T-cell immunogenicity have been investigated with a series of glycolipid analogs differing in their lipid or sugar moieties. These studies have shown that the intact structure of ceramide is important to confer T-cell immunogenicity. Furthermore, the type of the fatty acid and the presence of sphingosine tail also influence T-cell response. The sugar head of glycolipids is responsible for cognate interaction with the T-cell receptor (TCR), and modification of the type of glycosidic bonds- as well as of individual monosaccharides profoundly influence T-cell recognition. To investigate more in detail the CD1 molecular requirements for interaction with the TCR and the different glycolipids, we have drawn a model according to the crystal co-ordinates of mouse CD1.1 protein. This model has predicted which amino acids in both $\alpha 1$ and $\alpha 2$ helices direct their side chains towards the TCR. In addition, it has also identified four amino acids which point towards the CD1 groove and which may interact with the bound glycolipid. These amino acids form a sort of tweezers with the important function of holding the glycolipid ligand in the appropriate position. This model has been tested by generating 26 CD1a transfectants expressing CD1a molecules modified by site-directed mutagenesis. Mutagenesis in amino acids residing in the upper part of both $\alpha 1$ and $\alpha 2$ helices impair, with different efficiency, the reactivity of a series of CD1a-restricted T-cell clones. Importantly, all the mutants in the four amino acids contributing to the tweezers abolish T-cell recognition, thus confirming their important role in glycolipid antigen-presentation.

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*A*ntiganglioside IgM Autoantibodies, Ganglioside-mediated Immunosuppression, and Cancer Vaccines

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Carcinomas of the colon and pancreas selectively overexpress a variety of gangliosides. Elevated serum ganglioside levels in patients with colon or pancreatic cancer suggest that growing tumor cells release their surface gangliosides into the circulation. To examine this possibility, we measured serum ganglioside levels in patients undergoing cryosurgical ablation (CSA) of liver metastases from colon carcinoma. CSA is a surgical procedure in which a tumor is frozen and thawed *in situ*. Post-CSA serum ganglioside levels were higher than pre-CSA levels and remained elevated for more than three weeks. To verify antibody-mediated downregulation of serum gangliosides, we measured serum antiganglioside IgM levels before and after CSA. Patients undergoing CSA had elevated levels of specific antibodies to colon carcinoma-associated gangliosides GM2, GD1b and GT1b but not to normal tissue-associated gangliosides such as GM3 or GM1. This finding suggests that CSA-induced tumor necrosis, without any exogenous adjuvant, induces antiganglioside IgM to downregulate serum gangliosides. Antiganglioside IgMs are natural autoantibodies found in healthy individuals. When we tested the feasibility of augmenting these autoantibodies by vaccinating patients with exogenous membrane-bound gangliosides admixed with an adjuvant, we found that serum ganglioside levels postvaccination were inversely correlated with serum antibody titers. One of the tumor-associated gangliosides stoichiometrically bound to a CTL-stimulatory cytokine, indicating the immunomodulatory role of the ganglioside. Our observations suggest that antigen-targeted immunotherapies should attempt to restore immunocompetence by augmenting the levels of antiganglioside antibodies.

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*I*mmune Dysfunction in Chronic Disease: Molecular Basis and Implications for Immunotherapy

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Chronic diseases including cancer, autoimmune diseases and chronic infectious diseases such as leprosy and tuberculosis, are in part characterized by a dysfunctional immune response, which not only is not effective in controlling the progression of the disease, but also becomes part of the pathological process that leads to tissue damage. The changes in immune function have been repeatedly observed, but the basis for these changes has been poorly understood. Several mechanisms have been postulated to explain this diminished immune response. These include a lack of adequate antigen presentation and expression of co-stimulatory molecules, a preferential response of Th2 cytokines and the presence of alterations in the process of T-cell signal transduction. Our laboratory has focused its efforts on understanding the molecular basis leading to changes in the expression of T-cell signal transduction molecules, its possible clinical relevance and the means to prevent or reverse these changes. We will discuss possible mechanisms that lead to the down-regulation of the expression in T-cell signal transduction molecules with a resulting T-cell dysfunction and the preferential production of Th2 cytokines. In addition we will discuss possible means to measure these changes in patients and ways to prevent and/or reverse these signal transduction alterations. The data in this field suggests

that the changes in monocytes/macrophages populations and T cells are part of a continuous process, which ultimately leads to a dysfunctional immune response.

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*V*ery Small Size Proteoliposomes: Engaging Tumor Antigens with Innate Immunity

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Most tumor associated (TA) molecules are weakly immunogenic. Since 1993 we have been trying to improve weakly immunogenic TA targets through its association with strong innate immunity ligands. As a model we chose GM3 ganglioside associated to the outer membrane protein complex of *Neisseria meningitidis* to form very small size proteoliposomes (VSSP). On the other hand, murine B16 melanoma expresses GM3 and then constitutes an appropriate model for the development of GM3-related immunotherapeutic ideas. Previous immunization with GM3/VSSP vaccine increased the overall survival of mice challenged with B16 melanoma cells, whereas GM3/VLDL immunogen was ineffective. The antitumor effect of vaccination was observed not only by the enhancement of the host's capacity to reject the tumor but also by a reduction of B16 melanoma growth in tumor-positive animals. Two intriguing properties of the VSSP vaccine ability to induce protection were: i) a dependence on the ganglioside structure (NGcGM3 ganglioside is absent from B16 melanoma so its corresponding VSSP vaccine was unable to protect mice, even though this ganglioside is conformationally identical to GM3) and ii) their non-transient character measured in the experiments.

Immunization experiments in Balb/c and Balb/Xid mice suggested that B1 cells may regulate GM3/VSSP-induced specific IgG antibodies. Other group of experiments showed that VSSP and lipopolysacharides (LPS) induce similar dendritic cell (DC) maturation and stimulate the production of similar patterns of cytokines and, more strikingly, that the adjuvanticity of VSSP cannot be explained by the LPS component alone since VSSP induces IL12p40, IL1b, MIF and IL6 in C3H/HeJ mice which are hyporesponsive to LPS. Furthermore, DC matured by VSSP induced $CD4^+$ T-cell activation and INFg production in a transgenic OVA model.

These findings suggest that vaccination with weak antigens (GM3)/VSSP can induce a specific and long-lasting antitumor protection, most likely due to the exceptional capacity of this vehicle to mobilize innate immunity.

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*T*umor Growth Inhibition and Cell Cycle Effects of Antisense Oligodeoxynucleotides Targeted Against Thymidylate Synthase

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Tomudex and 5-fluorouracil are chemotherapeutic agents that target thymidylate synthase and are effective against a variety of tumors. Resistance to these drugs is often mediated by thymidylate synthase overexpression. We previously reported (Ferguson *et al*., Br J Pharmacol 1999;127:1777-86) that an antisense oligodeoxynucleotide targeting thymidylate synthase mRNA sensitized HeLa cells to Tomudex and 5-fluorodeoxyuridine *in vitro*. The aims of this study were to test the efficacy of antisense thymidylate synthase oligodeoxynucleotides against human colon cancer in nude mice, and to examine the mechanism of inhibition of cell proliferation. Growth of human HT29 colon tumors in nude mice treated with oligodeoxynucleotides was monitored, and levels of thymidylate synthase protein in the tumors determined. Distribution of radiolabeled oligodeoxynucleotides was ascertained in tumor and normal tissue sections. Apoptosis was measured in HeLa cells treated with oligodeoxynucleotides, and flow cytometry was used to examine cell cycle parameters. Statistical analyses were two-sided *t* tests. Thymidylate synthase antisense oligodeoxynucleotide localized to tumors, inhibited tumor growth when administered immediately after tumor implantation, and reduced thymidylate synthase levels in tumors. Antisense oligodeoxynucleotide did not affect levels of apoptosis in HeLa cells *in vitro*. However, accumulation of cells in G2/M was observed 24 to 48 hours after treatment. A G2/M cell cycle block induced by antisense thymidylate synthase oligodeoxynucleotide likely mediates reduced tumor growth *in vivo*, especially in microscopic tumors. Antisense thymidylate synthase oligodeoxynucleotide may be an effective *in vivo* therapy for colon carcinoma, and may provide a means to circumvent drug resistance.

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A Practical Approach to the Development of Vaccines for Clinical Use

AG Dalgleish, J Eton, J John, L Lowe, M Turner, H Pandha

The claimed improved survival in Stage IV disease of patients with melanoma who received three allogeneic cell lines together with BCG as pioneered by Morton and colleagues, led us to examine the practicality of this approach and its efficacy. Experiments in both murine and rat melanoma and prostate models clearly show that contrary to expectations, allogeneic cells improve the chance of survival of animals challenged with live tumor. We next investigated the role of gene transfer and a wide variety of adjuvants. Although a number of cytokines transfected into autologous cells increased the immune protection to tumor challenge, this does not appear to be the case in allogeneic cells which have their own adjuvanticity. Moreover, other non-specific adjuvants such as BCG and oil/water emulsion preparations can confer the same degree of adjuvanicity as gene transfected cells.

We are currently evaluating methods of enhancing the activity seen to date and methods include the use of the HSVtk suicide gene technology to enhance immune responses, tumor antigen presenting cell hybridomas, and the use of DNA vaccines together with dendritic cell preparations.

In addition to the above approaches, we are targeting the tumors defence mechanisms against an effective immune response and are developing an anti-idiotype strategy aimed at CD55 the DAF molecule, as the best correlate of protection with cell based vaccines appears to be complement dependant cytotoxicity. We now have a number of randomised and non-randomised adjuvant and therapeutic vaccine trials in melanoma, prostate, colorectal cancer, as well as pilot studies in renal, pancreas and CNS.

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Intratumoral Recombinant GM-CSF Encoding Vaccinia Virus as Gene Therapy in Patients with Melanoma

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Seven immunocompetent, revaccinated patients with stage IV melanoma underwent intratumoral (IT) treatment of dermal/SC metastases with injection (twice per week x 6 weeks induction) of escalating doses (10^4 - 2 x 10^7 PFU/lesion, 10^4 - 8 x 10^7 /session) of a Vaccinia/GM-CSF recombinant (RV). Patients 3 and 5 were maintained on treatment for 12 and 6 months respectively. Systemic toxicity was dose-related and limited to mild flu-like symptoms. Inflammation was seen with > 107 PFU RV. Chronically treated lesions showed a dense infiltration with CD4+ and CD8+ lymphocytes, histiocytes and eosinophils. In all 5 patients who received the highest doses of RV, treated lesions regressed. Three (3, 5, 7) had regression of all injected lesions and untreated regional dermal/SC metastases. All 7 Patients manifested serum antibody responses to vaccinia and to beta-galactosidase secondary to lac-Z expression in the RV. Using RT-PCR and primers specific for the vaccinia thymidine kinase gene, virally encoded mRNA was seen beginning at 6 hrs and continuing to, at least, three days following treatment (both early and late in the treatment course). Using primers specific for Vaccinia passenger gene encoded GM-CSF, mRNA was found at 18 and 48 hrs after injection in both acute and chronically treated lesions. These studies demonstrate that: (1) recombinant Vaccinia viruses are an effective vector for *in situ* gene transfer, (2) developing immunity to Vaccinia proteins does not block effective tumor infection/transfection and (3) treated and untreated regional dermal/SC metastases regress.

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Treatment of Cancer in the Adjuvant Setting with a Polyvalent, Antibody-inducing Vaccine

P Livingston

Studies in experimental animals demonstrate that passively administered or vaccine induced antibodies can eliminate circulating tumor cells and micrometastasis, an extent of tumor similar to that seen clinically in the adjuvant setting. We have identified a series of cell surface glycolipid antigens [(GM2, GD2, GD3, Fucosyl GM1, globo H and Lewis Y (Le^Y)], glycoprotein (mucin) antigens (Tn, sialyl Tn (sTn), Thomsen-Friedenreich antigen (TF) and MUC1), polysialic acid and a protein antigen (KSA) on a variety of cancers as suitable targets for antibody attack. The optimal vaccine formulation involves chemical conjugation of the antigen, the antigen lactone (L) or an antigen cluster (c) to a carrier molecule (keyhole limpet hemocyanin (KLH) has proven optimal) and the use of a potent immunological adjuvant. The saponin immunological adjuvants (QS-21 and GPI-0100) obtained from the bark of a South American tree *(Quillaja saponaria)* have been the most potent adjuvants both for antibody induction against these antigens and for induction of interferon gamma against KLH. To date, antibodies have been induced in the majority of patients vaccinated with single antigen conjugate vaccines against each of these antigens, except for polysialic acid and KSA, and against tumor cells expressing these antigens. IgM and IgG antibodies capable of reacting with these antigens on tumor cells (FACS) and activating complement (IA) were induced in most patients, as summarized below. Complement mediated cytotoxicity (CDC) was detected with antibodies against glycolipid but not against mucin antigens.

Pilot trials using polyvalent KLH-conjugate vaccines against GM2, Globo H, Le^Y, sTn, TF and MUC1 for patients with breast or ovarian cancer, the same 6 antigens plus Tn for prostate cancer patients, and GM2, GD2 and GD3 for patients with melanoma or sarcoma are being initiated to ensure safety and immunogenicity. Phase III double blind randomized trials in the adjuvant setting comparing specific immunization with the polyvalent KLH conjugate vaccines to nonspecific immunization with KLH and QS21 alone are planned for 2001 (Table).

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Table. Summary of serological results in vaccinated patients.

Vaccine antigen	No. of patients	Median ELISA Pre/post		IgG subclass	Median FACS Pre/post		Median IA post	Median CDC Pre/post
		IgM*	IgG		IgM	IgG		
Fucosyl GM1	10	0/320	0/640	IgG1	11/84	10/33		10/71
GM2	100	0/640	0/320	IgG1+3	11/65	10/41	++	2/44
GD2	30	0/40	0/160					
GD3L	12	0/40	0/160		9/10	10/29	+	3/14
Globo H	30	0/640	0/40	IgG1+3	10/25	10/13	++	4/36
Lewis Y	18	0/80	0		7/23		+	3/26
Tn(c)	15	0/2560	0/1280					–
STn(c)	27	0/1280	0/160	IgG3	6/30	10/8	+	–
TF(c)	15	0/320	0/10		11/23	10/25	+	–
MUC1	45	0/1280	0/5120	IgG1+3	11/51	11/25	+	–
Polysialic acid	5	0/0	0/0					
KSA	15	0/40	0/160		10/12	11/13	–	–

0 = titer less than 1/10

Epidermal Growth Factor-based Vaccine: Results of Clinical Trials in Patients with Epidermoid Origen Tumors

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Epidermal Growth Factor (EGF) promotes cellular proliferation on binding to its membrane receptor (EGF-R). Anti-EGF antibodies raised by active immunization

with EGF should "block" this binding, inhibiting the resulting proliferation mechanisms. In pre-clinical studies, we demonstrated that immunization with EGF, in mice and monkeys, provokes an anti-EGF antibody (Ab) response and that immune sera block the binding of EGF to its receptor in an *in vitro* assay. EGF-immunized mice showed a significant increase in survival when transplanted with EGF-R-expressing tumors, as compared with non-immunized tumor-transplanted controls. In a first small pilot clinical trial, we demonstrated the immunogenicity and lack of toxicity of a vaccination with human EGF (2 doses) in 10 patients with advanced epidermoid carcinomas. Data pooled from two Phase I randomized trials of EGF vaccination in 40 non-small cell lung cancer (NSCLC) patients with advanced disease, considered ineligible for other standard anti-cancer treatment, are now available. A 5 dose protocol was used and patients to be included were randomized to 4 treatment arms: with or without a low dose of cyclophosphamide (CPM) before vaccination, and with alum or Montanide ISA 51 as adjuvants. The 40 vaccinated patients, 36 showed seroconversion (at least a doubling of pre-vaccination EGF antibody levels). According to the anti-EGF antibody response, patients were classified as good antibody responders [GAR, those reaching Ab titers of at least 1:4000 (serum dilution) and at least 8 times the pre-vaccination titer] and poor antibody responders (PAR). Pre-treatment with CPM provoked an increase in Ab titer levels but not an increase in the percentage of GAR. The use of Montanide as adjuvant significantly increased antibody responses and the percentage of GAR. There were no significant differences in survival times (SV) between randomized groups. In all patients treated, GAR showed a significant increase in survival (n = 19, median SV 9.1 months) as compared with either PAR (n = 21, median SV 4.5 months) or with a historical control group (n = 29, median SV 5.6 months) (p = 0.02). All treated patients (GAR+PAR) showed a tendency to increase in survival (n = 40, median SV 8.17 months) as compared with historical controls. Vaccination was safe; no evidence of severe clinical toxicity was observed.

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Antibody-based Therapy

*U*se of the Humanized Anti-EGF-R Monoclonal Antibody h-R3 and Radiotherapy in the Treatment of Advanced Head and Neck Cancer Patients

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Squamous cell carcinomas of the head and neck (SCCHN) are characterized by over-expression of epidermal growth factor receptor (EGF-R). Ior egf/r3, a neutralizing murine monoclonal antibody (MAb) against EGF-R was generated at the Cuban Institute of Oncology. The antibody recognizes EGF-R with high affinity, inhibiting tyrosine kinase activation. Immunoscintigraphy in 148 patients with this 99-m technetium labeled MAb, showed an overall sensitivity and specificity of 84.1% and 100 %, for *in vivo* detection of epithelial tumors. The antibody was humanized (h-R3), exhibiting similar capacity to inhibit EGF binding to EGF-R. In a previous trial, the low toxicity and immunogenicity of h-R3 was demonstrated. To evaluate the efficacy and safety of h-R3 at multiple doses, in combination with radiotherapy in advanced SCCHN patients, a phase Ib/IIa trial was conducted. Twelve patients with advanced loco-regional SCCHN who were suitable candidates for radical radiation therapy were included in the trial. Patients received six weekly intravenous doses of h-R3 at 4-dose levels in combination with radiotherapy. Single MAb doses ranged from 50 to 400 mg. Before and after treatment, a small biopsy of primary tumors was taken to evaluate EGF-R expression, proliferative index and blood vessel staining by immunohistochemistry. EGF-R over expression was considered an inclusion criterion.

After MAb treatment, main adverse events consisted in fever, hypotension, somnolence, tremors, and disorientation. Irradiation toxicity was not exacerbated by the addition of h-R3. Seven out of 12 patients showed an objective response (6 complete and 1 partial remission). Complete responses were achieved at the 4-dose levels: 50 and 100 mg (1 patient for each dose), 200 and 400 mg (2 patients). In histological studies, tumor sections before treatment showed high vessel density, while treated tumors were characterized by a remarkable reduction of vascularity. EGF-R expression and proliferative index significantly decreased after treatment completion in responding patients.

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*T*hree-step Radioimmunotherapy with ^{90}Y-biotin: Dosimetry in Cancer Patients

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This report presents the results of pharmacokinetic and dosimetric studies obtained in 24 cancer patients treated by a three-step avidin-biotin approach as pretargeting system in radioimmunotherapy (RIT). Special consideration was given to the dose delivered to the red marrow and to the haematological toxicity. The protocol consisted in the injection of biotinylated MAbs (first step) followed one day later by the combined administration of avidin and streptavidin (second step). After 24 h, biotin radiolabeled by the DOTA chelating agent with 2-6 GBq of ^{90}Y was injected (third step). ^{111}In-biotin was used as a tracer of ^{90}Y to follow the biodistribution during therapy. Serial blood samples and complete urine collection were obtained over 3 days. Whole body and SPECT images taken at 1, 16, 24, 40 h after injection were used to extrapolate ^{90}Y-biotin time-activity curves. A compartmental model was used to calculate the residence time values (t) for critical organs and tumor; the absorbed doses were estimated using MIRDOSE3.1 software.

The mean t value in blood was 2.0 ± 1.1 h; the mean urinary excretion in the first 24 h was 82.5 ± 10.8 %. Kidneys, liver, bladder and red marrow mean absorbed doses were: 1.62 ± 1.14; 0.27 ± 0.23; 3.61 ± 0.70; 0.11 ± 0.05 mGy/MBq respectively; the dose to the tumor ranged from 0.62 to 15.05 mGy/MBq. Pretargeted 3-step RIT allows the administration of high ^{90}Y activities capable of delivering a high dose to the tumor and sparing normal organs and red marrow. Although ^{90}Y-biotin rapidly clears from circulation, accurate quality controls of the radiocompound are recommended, as small fractions of free ^{90}Y originating from a non-complete radiolabeling can significantly contribute to red marrow dose (3.26 mGy per MBq of free ^{90}Y) and may explain some high levels observed for haematological toxicity.

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Antibodies, Antigen Mimicry and Active Specific Immunotherapy of Melanoma

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Like many tumor associated antigens identified in human malignant cells, the human high molecular weight-melanoma associated antigen (HMW-MAA) is a self-antigen. To overcome unresponsiveness to HMW-MAA, we have been testing the strategy to utilize mimics of HMW-MAA as immunogens. In previous studies we have shown that the mouse anti-idiotypic (anti-id) MAb MK2-23 which mimics the determinant recognized by mouse anti-HMW-MAA MAb 763.74 can induce humoral anti-HMW-MAA immunity in about 60% of patients with melanoma. The reactivity of anti-anti-id antibodies with melanoma cells is low, although that with the immunizing anti-id MAb is high. This finding is likely to reflect the lower association constant of the anti-anti-id antibodies for the HMW-MAA than for the immunizing anti-id MAb. The association between the anti-anti-id response and an improved clinical course in patients with melanoma has prompted us to continue these studies. To overcome some of the limitations of the use of mouse anti-id MAb as immunogens, i.e. inability to induce HLA class I antigen restricted, HMW-MAA specific CTL, induction of anti-mouse Ig antibodies, we are testing peptide mimics of HMW-MAA as immunogens. Peptide mimics have been isolated by panning two phage display peptide libraries with mouse anti-HMW-MAA MAb and with human anti-HMW-MAA scFv fragments. Most of the isolated peptides inhibit the binding of the corresponding antibody to the HMW-MAA in a dose dependent fashion, but do not share sequence homology with that of the HMW-MAA. Peptide mimics have been found to elicit anti-HMW-MAA antibodies and DTH to HMW-MAA bearing melanoma cells. The level of anti-HMW-MAA antibodies elicited by mimics of HMW-MAA is low, but can be markedly enhanced by boosting the host with the original antigen. These findings suggest that sequential immunization with a mimic of the antigen and with the original antigen may be an effective strategy to elicit a strong immune response to a self-tumor associated antigen.

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Anti-idiotype Vaccine Therapy of Colorectal Cancer Targeting the Carcinoembryonic Antigen

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Our goal is to use CEA as a target for immunotherapy in CEA-positive cancer patients who are all immune tolerant to the native antigen. We isolated and characterized an anti-idiotype (Id) monoclonal antibody 3H1 designated CeaVac, which mimics a distinct and specific epitope of the M_r 180,000 CEA and can be used as a surrogate for CEA. Active immunization of different species of animals such as mice, rabbits and monkeys with 3H1 results in a polyclonal anti-CEA response as well as T-cell proliferative responses against CEA. Preclinical studies demonstrating the ability of 3H1 to stimulate tumor-specific protective immunity in a murine model using CEA-transfected MC38cea tumor cells confirmed the potential role of 3H1 in anti-Id therapy. Our initial clinical trials of 3H1 adsorbed to aluminum hydroxide (alum) have shown that 3H1 can break immunetolerance to CEA, and patients with advanced metastatic disease who generate an active specific immunity to CEA may have a survival benefit. In this phase Ib trial, hyperimmune sera from 17 of 23 patients demonstrated an anti-anti-idiotypic Ab3 response, and 13 of these responses were demonstrated to be true anti-CEA responses (Ab1'). The antibody response was polyclonal, and 11 mediated antibody-dependent cellular cytotoxicity. Ten patients had idiotypic T-cell responses, and five had specific T-cell responses to CEA. Toxicity was limited to local swelling and minimal pain. Next, we treated 32 patients with surgically resected colon cancer with CeaVac (3H1). We also compared the immune responses between patients treated with fluorouracil (5-FU) chemotherapy regimens plus vaccine versus vaccine alone. All patients entered onto this trial generated high-titer IgG antibody and T-cell proliferative immune responses against CEA and the peptides LCD-2 and CEA-B derived from 3H1/CEA sequence homology. The 5-FU regimens did not have a qualitative or quantitative effect on the immune response. A number of very high-risk patients continue on study. These data warrant a phase III trial for patients with resected colon cancer.

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Immune Responses in Melanoma Patients Immunized with an Anti-idiotype Antibody Mimicking N-glycolyl Containing Gangliosides

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We have generated an anti-idiotype monoclonal antibody (1E10) specific to an Ab1 monoclonal antibody able to specifically react with N-Glycolyl-containing gangliosides and with antigens expressed on melanoma and breast cancer. A pilot clinical trial for twenty patients with advanced malignant melanoma have been carried out. Pa-

tients received six doses of 2 mg of aluminum hydroxide-precipitated 1E10 monoclonal antibody, injected intradermally into multiple sites at two week intervals. All patients had stage IV malignant melanoma, the median age was 57 years (range 29-76 years), and the male:female ratio was 7:12. Fourteen patients completed the entire vaccination schedule, five patients were removed from the study because of disease progression and one patient was lost to follow-up. No evidence of serious or unexpected adverse effects have been observed in this clinical trial. Main cases of toxicity included erythema and induration at the injection site, sometimes associated with mild pain and low grade fever (WHO grades I, II). All cases of toxicity resolved spontaneously, lasting from 3 to 7 days. Chills and mild cephalea occurred in few patients. The median survival was 42 weeks with 5 patients surviving for more than one year. Serum was obtained at baseline and before each immunization. The presence of human anti-mouse antibodies (HAMA), specific anti-1E10 and anti-NeuGcGM3 antibodies was evaluated before treatment, fourteen days after each immunization. Hyperimmune sera from sixteen of the seventeen patients who received at least four doses of the vaccine revealed an anti-anti-idiotype (Ab3) response as demonstrated by the inhibition of Ab2 (1E10 Monoclonal antibody) binding to Ab1 (P3 Monoclonal antibody). Specific antibody responses were observed both against the 1E10 antibody and the NeuGcGM3 ganglioside. The specificity of the immune response was confirmed by high performance thin layer chromatography-immunostaining using a panel of standard gangliosides. The antibody response against monoclonal antibody 1E10 was predominantly of the IgG isotype, with antibody titers ranging from 1:10,000 to 1:100,000, and the anti-ganglioside response was of IgG and IgM isotypes showing titers up to 1:3200 (IgG) and 1:12800 (IgM). The results of this clinical trial have demonstrated that this 1E10 anti-idiotype vaccine was safe, well tolerated and immunologically effective, with most patients being able to generate a specific immune response against 1E10 and NeuGcGM3 ganglioside.

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Compared Immunogenicity in mice of GD3 Ganglioside Purified from Human Malignant Melanoma Versus Glyco-replica Peptides Mimicking GD3

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Glyco-replica peptides mimicking GD3, that is the major ganglioside of human malignant melanoma, were isolated from a phage-displayed peptide library with a mouse monoclonal IgG3 antibody (MAb) specific for GD3 ganglioside. Sequencing of the peptides showed that they were made of 15 to 20 amino acids. The immunogenicity of peptide R4, the most reactive peptide to MAb antiGD3, was studied following immunization of mice with the peptide R4 coupled to keyhole limpet hemocyanin (KLH), and the immune response was compared to that obtained with pure ganglioside GD3. All immune sera displayed specific IgG and IgM antibodies reacting with both GD3 and R4 peptide, as shown by ELISA and immunostaining on PVDF membranes. However, the IgG subtypes were quite different with respect to the antigen tested. Mice immunized with R4 had high titers of specific IgG3 when tested on GD3, whereas the titers of IgG3 bound to R4 were weak. Mice immunized with GD3 had high titers of IgG2a and IgG1 specific for both GD3 and R4. Thus, peptide R4 induced IgG3 antibodies with a greater affinity for GD3 than for the peptide itself. A study by flow cytometry on murine melanoma cells expressing GD3 showed that, although the binding of immune sera was stronger for mice immunized with GD3, the sera of mice immunized with R4 also reacted with these cells. These results suggest that glyco-replica peptides mimicking gangliosides may be useful to trigger an immune response against tumor cells such as human malignant melanocytes expressing those gangliosides. A study is in progress to determine which amino acids of the peptides are critical for mimicking GD3.

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Phage Display for the Isolation of Therapeutic Antibodies: Applications and Improvements

S Dübel

Nine years after the invention of the antibody phage display method, the first human antibodies isolated from libraries have already entered the clinics. However, there is still potential to increase the performance of the selection technology. We have constructed a novel Helperphage ("Hyperphage®"), which allows an effective packaging of phagemids, yielding close to 100% of phage which carry antibody fragments, compared to less than 1% achieved with M13KO7 or other common helperphage. The antibody phage made with Hyperphage showed a stronger binding to their antigen, and a 400 fold increase in the number of functional phage. This allows to package large libraries much more efficiently, by avoiding that the majority of packaged phages cannot contribute to the panning enrichment since they do not present an antibody on their surface. Further, Hyperphage packaging dramatically increased the probability to get binders in the first panning step, by shifting the ratio between the few specific antibody phage and the antigen towards binding. This effect has been demonstrated by panning a universal human antibody library with tetanus toxin. After just two rounds of panning, more than 50% of the eluted clones bound strongly to the antigen. Conventional M13KO7 helperphage packaging of the same library did result in 3% positive clones which included no strong binder. Antibodies with therapeutic potential can be isolated from immunized donors. We used this approach to isolate antibody fragments binding to the surface of intact Hantavirus particles. Hantavirus is the causative agent of Hemorrhagic Fever with Renal Syndrome (HFRS), a life threatening disease with a worldwide distribution. Most cases are reported from China. We used blood

samples from patients which had survived the infection and constructed antibody display libraries. A panel of different antibodies to the two surface glycoproteins was obtained. For the expression of therapeutically active complete IgG molecules, we constructed a cassette Baculovirus vector system which allows the direct insertion of the antigen binding regions obtained by phage display. Yields up to 18 mg/L allow convenient production of material sufficient for animal tests.

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*M*olecular Farming of Clinically Relevant Human Recombinant Antibody Fragments

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Antibodies are the paradigm for the construction of high affinity, protein-based binding reagents. They offer the opportunity to design highly potent molecules for the rapid diagnosis and therapy of many severe diseases. Clinical applications of antibody fragments cover the full range of diagnostic *in vitro* immunoassays through to therapeutic *in vivo* tumor imaging and targeting reagents. The enormous potential of human antibodies for improving human health will create a need for the production of inexpensive, safe, highly potent, novel recombinant human antibodies for e.g. tumor imaging and targeting. The strength of our approach is that we intend to combine the power of developing and optimizing human antibodies through phage display, with the almost unlimited production potential of using plants as bioreactors for producing 2nd and 3rd generation human antibodies. Plants produce functional, safe recombinant antibodies on a large scale at low cost. The absence of pathogenic vector or oncogenic sequences in the plant expression system is a tremendous advantage, which will have a major impact on the development of improved, efficacious and safe diagnostics, therapeutics and vaccines.

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Autoimmunity and Regulation of the Immune System

*D*NA Immunization for Autoimmune Disease

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DNA vaccination is an effective means of protecting experimental animals against infectious pathogens and cancer, and has more recently been used to prevent autoimmune disease. The mechanism involved in the immune response to microbial DNA are not clear but several experiments point to a paradox in which antigen in the DNA form can down regulate autoimmune damage but boost the attack to foreign pathogens. We are presenting our recent experiment in murine models of autoimmune disease in order to discuss the application to human disease. We will address the results of DNA immunization with insulin DNA in the NOD mouse as well as the use of myelin antigen for the prevention and treatment of experimental autoimmune encephalomyelitis.

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*T*herapeutic Application of Mutant Forms of Bacterial Superantigens

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Bacterial superantigens are characterized by their ability to interact with and activate T cells that share defined T-cell receptor Vβ segments. There is increasing evidence that superantigens are involved in the pathogenesis of several autoimmune diseases such as psoriasis. This pathogenic mechanism has been experimentally reproduced in a SCID-hu xenogeneic transplantation model. We analyzed the effects of different bacterial superantigens on the induction of psoriasis in this model. Staphylococcal enterotoxin B and exfoliative toxin triggered the onset of psoriasis when administered repetitively i.e. over a period of two weeks, whereas staphylococcal enterotoxin A (SEA) representing a distinct subfamily of staphylococcal enterotoxins only mimicked certain aspects of psoriasis. The biological effects of SEA were more pronounced when a mutated form, SEAH187A, of this superantigen with reduced affinity to MHC class II was co-injected. Another mutated variant, SEAF47A/D227A, exhibiting no measurable MHC class II affinity blocked the effects triggered by wild-type SEA when injected in a 10-fold higher dose. Inhibition was specific since induction of psoriasiform epidermal changes by SEB could not be blocked. Since, in contrast to the other superantigens tested, SEA is capable of inducing epidermal thickening but not the typical appearance of psoriasis we conclude that bacterial superantigens may differ with regard to their effects on human non-lesional psoriatic skin. SEA-mediated effects were blocked by a genetically engineered superantigen highlighting the potential therapeutic use of mutated superantigens. In the field of oncology, SEA application for therapeutic purposes has already been undertaken in the form of fusion proteins composed of tumor-reactive monoclonal antibodies and the superantigen.

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*B*acteriophages as Anti-bacterial Agents and Immunomodulators

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Immunocompromised patients are prone to life-threatening infections; therefore, their appropriate treatment is of utmost importance. Bacteriophages (BP) are viruses that infect bacteria, multiply within them and lyse them. At our Institute, we have treated with BP approx. 1500 patients with antibiotic-resistant bacterial infections with a success rate exceeding 90%.

Recent emergence of bacteria resistant to all available antibiotics poses a major danger to human health and threatens to return us to a pre-antibiotic era. In this sense, our findings with BP as anti-bacterial

agents are becoming of increasing interest. Interestingly, our *in vitro* and *in vivo* data indicate that BP can modulate the immune response and upregulate immunity in immunocompromised individuals.

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The Complement Attenuating Effect of Intravenously Applied Human IgG is Primarily due to Naturally Occurring Anti-C3 Antibodies

HU Lutz, P Stammler, V Bianchi, M Schlumberger, A Luginbohl, PJ Spoth, R Troeb, E Jelezarova

High dose, pooled, human IgG has beneficial effects, when intravenously applied to patients (1-2 g per kg body weight) with inflammatory, complement-mediated diseases. IgG attenuates complement activation *in vitro*, because it stimulates factor I-dependent inactivation of those C3b-containing complexes that best amplify complement deposition (C3b2-IgG complexes) (Lutz *et al*. Blood 88 [1996] 184). $C3b_n$-containing complexes could also be verified in rapidly denatured plasma of patients with dermatomyositis. Infusion of high dose IgG lowered the steady state concentration of these complexes significantly to a level of 50% by day 14. As judged from *in vitro* experiments, the complement-attenuating principle was retained by F(ab')2 fragments, implying the involvement of certain naturally occurring antibodies (NAb). Thus, we purified anti-C3 NAb by affinity chromatography of pooled human IgG on immobilized C3. F(ab')2 fragments of these NAb were up to 1000 times more effective than those from whole human IgG, in stimulating inactivation of $C3b_2$-IgG complexes which were generated in 20% serum in which classical pathway complement activation was induced. They stimulated inactivation of $C3b_2$-IgG complexes at 1-20 µg/mL in a dose-dependent way, while $F(ab')_2$ of whole IgG had no effect at corresponding concentrations. Anti-C3 NAbs were effective, when supplemented alone or together with 0-5 mg/mL of exogenous IgG depleted for anti-C3 NAbs. Since anti-C3 NAbs reduced the effective concentration of native $C3b_2$-IgG complexes and the total amount of these complexes generated over an incubation period, they reduced the total extent of C3 activation by 25 ± 5% at 5 µg/mL with no further reduction at higher concentrations. The effect of anti-C3 NAbs was not abrogated by a pretreatment with diisopropyl-fluorophosphate. Their attenuating potential could also be verified on an artificially generated alternative C3 convertase. Anti-C3 NAbs, but not IgG depleted of anti-C3 NAbs, lowered the number of active C3 convertases, when added during C3 convertase assembly on $C3b_2$-IgG complexes. Hence, anti-C3 NAbs appear to regulate the amplification loop by stimulating inactivation of $C3b_2$-IgG complexes which represent its major activators (Jelezarova and Lutz, Mol Immunol 1999;36:837). In an ongoing project with J.A. Schifferli and PJ Spoth we investigate whether these NAbs even counteract autoaggressive antibodies that stabilize C3 convertases, as in membranoglomerulonephritis.

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How Preexisting, Germline-derived Antibodies and Complement May Help Induce a Primary Immune Response to Non-self

HU Lutz, A Luginbuhl, E Jelezarova

Preexisting, germline-encoded antibodies (naturally occurring antibodies, NAb) bind to conserved epitopes on invading non self-antigens and form immune complexes that initiate complement deposition (Thornton *et al*. Clin Exp Immunol 1996;104:531). Nascent C3b may preferentially bind to NAb rather than to antigen of the immune complex, since IgG has an affinity for C3 which can be higher for some NAbs (Lutz *et al*. J Biol Chem 1993;268:17418). C3bs are ester bonded to each other and to the NAb in these complexes. They remain associated with the non-self antigen (C3b-C3b-NAb....non self-antigen). Once C3b-C3b-Ig complexes are inactivated to C3d or C3dg, they retain dimeric C3 fragments, as we have verified on artificially generated complexes. Thus, (C3dg-C3dg-NAb....non self-antigen) complexes may bind bivalently to B cells via complement receptor 2 (CR2). In some cases CR2-bound (C3dg-C3dg-NAb...non self-antigen) complexes may further be recognized by Ig determinants on B cells, whereby an immune response is elicited. Since conserved epitopes on the non-self-antigen are already occupied by NAbs, only B cells specific for non self-epitopes would be stimulated by complex-induced coaggregation of such an Ig determinant with CR2. This can explain directed affinity maturation towards non self and protection from an autoaggressive immune response to conserved epitopes. As NAbs exist at low concentrations, high dose free antigen will compete with the described complexes for Ig determinants on B cells, whereby unresponsiveness is induced. This may explain why antibody-production is bell-shaped, when plotted against antigen concentration. Down regulation of B cells may then be initiated by CR2-bound complexes which have lost the weakly associated non self-antigen and present C3dg-C3dg-NAb to Ig determinants. These complexes may trigger B cells specific for Ig, whereby an anti-Ig production is elicited (anti-hinge region: Terness *et al.* J Immunol 1995;154:6446) which follows any immunization and stops B-cell proliferation by cross-linking FcR to antigen-carrying Ig determinant (Diegel *et al.* J Biol Chem 1994;269:11409).

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Natural Human Antibody and Intravenous Immunoglobulin Regulation of Activated Cells

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Evidence from several *in vivo* and *in vitro* syngeneic murine tumor models of natural resistance supports the idea of polyclonal natural antibody (NAb) surveillance of developing tumors, preneoplastic and

PKC-activated cells. T lymphomas treated with the tumor promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to generate variants and repeatedly selected for high serum NAb IgG- plus IgM-binding, exhibited a reduced SC tumorigenicity of threshold tumor inocula. The selected cells bound monoclonal antibodies against activation-associated cell surface molecules including CD25 the IL2-R" chain and asialo forms of CD45RA a transient marker of T-cell activation. Thus, NAb may also regulate T-cell activation. These observations raised the possibility that direct down-regulation of T-cell activation may contribute to the beneficial effects of intravenous immunoglobulin (IVIG) against inflammatory and autoimmune diseases in humans. In order to identify the T-cell surface targets of IVIG, we have produced model human T cells through TPA treatment of Jurkat T-leukemia cells with repeated selection for high serum IgG- plus IgM-binding which mimics the conditions for IVIG action in patients. Cells produced from the 3rd and 4th sequential selections exhibited stable increases in serum IgG- plus IgM-binding of 28% for J3.1 and 45% for J4.1. The J4.1 bound at least 45% more IVIG than the parental cells consistent with their increased binding of pooled human IgG plus IgM. The J4.1 exhibited a 120% increase in expression of CD45RA. These data provide the first evidence that human serum natural IgG plus IgM antibodies and IVIG react with a CD45RA epitope. The conserved nature of many NAb and the contribution of endogenous NAb and passively administered IVIG to the control of immune activation, raise the possibility that the epitope on CD45RA may be a highly conserved homologous epitope or homotope of the immune system involved in health and disease. *Supported by the Manitoba Medical Services Foundation and The Bayer, Canadian Blood Services, Héma Québec Partnership Fund.*

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Ectopic Germinal Centres in Autoimmune Disease: A Key to Unlock the Autoimmune Response?

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Clusters of B and T cells, often with the histological appearance of germinal centres, are commonly found within the target tissues of autoimmune diseases such as synovial membranes in the joints of patients with rheumatoid arthritis or reactive arthritis, the thyroid in Hashimoto's thyroiditis, the salivary glands in Sjögren's syndrome and the thymus in myasthenia gravis patients. Follicular dendritic cells are intimately associated with these clusters of B and T cells. Many of the thymic germinal centres of myasthenia gravis patients stain with acetyl choline receptor showing that they are a source of autoantibody-producing B cells. We have analyzed the B-cell response in these structures in patients with Sjögren's syndrome and myasthenia gravis by microdissection, Ig V-gene cloning and sequence analysis. In each case, we have demonstrated that a germinal centre-type response is taking place, with antigen-driven, clonal B-cell proliferation, somatic hypermutation and affinity selection. We have reconstructed the antigen receptors of these germinal centre-like B cells and will use them to investigate the effects of somatic mutations on specificity and affinity for self-antigen, and to test the antigen mimicry hypothesis.

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Immunogenetics of Pediatric Autoimmune Hepatitis

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Type 1 Pediatric Autoimmune hepatitis (PAH) is a progressive liver disease characterized by the presence of circulating antinuclear (ANA) and/or anti-smooth muscle (SMA) autoantibodies, hypergammaglobulinemia and response to immunosuppressive treatment. We have recently demonstrated that PAH and adult autoimmune hepatitis (AAH) have different genetic susceptibility and clinical characteristics. Within AAH patients, studies in North America, and England demonstrated that AAH is associated with HLA-DR3 or HLA-DR4, meanwhile in Mexico, Japan and Argentina susceptibility to AAH is associated with HLA-DR4 alleles. In contrast, PAH patients in Argentina and Brazil are strongly associated with HLA-DRB1*1301. It was also found that DRB1* allele 1302, which only differ from DRB1* 1301 at position 86 of the DRβ chain confers protection for PAH. Following the hypothesis that Hepatitis A virus (HAV) infection may be a putative trigger for the autoimmune hepatitis, we investigated wether HLA may also influence the outcome of an HAV infection. We HLA-typed 67 children with self-limiting and 39 with protracted form (PF) of HAV infection. The uncomplicated forms show no significant increase of any HLA class I or II alleles. In contrast, DRB1*1301 was present in 46,1% of the children with PF (Vs 9.8% in healthy controls; RR:7.6, X^2 33.3, $p = 2x10^{-9}$). Uncomplicated hepatitis also developed anti-SMA/actin antibodies in 45% of the infected children, but only one child had detectable antibodies three months after the infection onset. In contrast, 69% of the patients with protracted forms still had anti-SMA/actin antibodies (titres ranged between 1:40-1:160) one year after the disease onset. Although 2 patients developed a Hashimoto's thyroiditis, none of them developed autoimmune hepatitis during a 5-years follow-up. These findings indicated that DRB1*1301 confers a higher susceptibility for prolonged forms of HAV infection with a sustained release of liver self-antigens. However, as they did not developed PAH we concluded that other still unknown susceptibility genes are required for the full development of the pediatric autoimmune hepatitis. We next investigated the cytokines involved in this disease. CD4+ T cells activated by HLA class II antigens can differentiate into a functional subset termed T helper 1 (TH1)-type and T helper 2 (TH2)-type cells, identified by interferon (IFN)-γ and interleukin (IL)-4, respectively. Similar cytokine profiles have also been described among CD8+ cells, named as type-1 cytotoxic and type-2 cytotoxic cells. IL-12, which is produced by antigen-presenting cells such macrophages or dendritic cells has emerged as a central cytokine in determining the outcome of the effector TH response. It was demonstrated that CD4+ T cells require IL-12 not only for differentiation into functional TH1 cells but also to sus-

tain memory/effector TH1 cells sufficient to mediate a biological outcome. Commitment to the Th1 lineage is in turn enhanced by IFN-γ, which maintain the high expression of IL-12R while inhibiting the growth of Th2 cells . IL-12 was also shown to play a pivotal role in the TH1-dependent mouse liver injury. Although normal human hepatic activated lymphocytes are able to produce IFN-γ, TNF-α, and/or IL-4, it can be assumed that a sustained presence of IL-12 should be present to induce a long lasting biological effect. We demonstrated that children with autoimmune hepatitis up regulated IL-12, IL-12R, IL-18, and IFN-γ mRNAs. Particularly interesting was the up regulation of the IL-12 β2 chain. This chain is not detected in normal liver, but in contrast to the IL-12α, that is constitutively expressed, the IL-12R β subunit became expressed after the activation of T cells. Both IL-2 and IL-12 are required for the induction of Th1 differentiation. The expression of IL-12 is known to inhibit the differentiation of activated naïve T cells towards a Th2 phenotype. It is also known that IL-18 acts synergistically with IL-12 in inducing IFN-γ from T cells undergoing differentiation to Th1 cells. Our study demonstrated that IL-12 and IL-18, was up-regulated both at the mRNA and at the protein level. Several findings suggest that DCs are likely to have a role in the pathogenesis of autoimmunity. Immature myeloid DC like the ones resident in the liver are deficient in cell surface costimulatory molecules, and can exhibit tolerogenic properties. It would be expected that development of liver autoimmunity will require the maturation of (DCp) precursors into a mature DCs able to prime Th1 cells via the release of IL-12 by DCs. This maturation occurs in response to inflammatory signals evoked by the interaction of pathogens with the innate immune system. We speculate, that the lost of liver tolerance and development of autoimmune hepatitis could be associated with an induced maturation of DCp, resulting in the up regulation of IL-12, IL-12R and IL-18, which in turn induced the sustained production of IFN-γ associated with the liver damage.

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Mathematical Modelling and *In Vitro* Proliferation Assays Provide Insights on the Mechanism of Linked Suppression

J Carneiro

Evidence for T-cell–mediated suppression has been derived from adoptive transfer experiments where the tolerant state of an animal is transferred to a naïve recipient by a particular subset of regulatory CD25+ Th cells. These regulatory Th cells also suppress the proliferative response of naïve CD25- Th cells *in vitro*. Recent evidence suggests that the suppression requires linked recognition of antigen and simultaneous conjugation of both regulatory and naïve T cells with a single antigen-presenting cell (APC). Despite this strong requirement, it is not yet clear what is the nature of the signals exchanged between regulatory and naïve cells in multi-cellular conjugates, and what are the consequences of these interactions for dynamics of the two cell populations. In this work we investigate this issue by a mixed modelling and experimental approach. We propose a general mathematical formalism describing the formation of multi-cellular conjugates, from where we derive particular models representing alternative mechanisms of T-cell–mediated suppression. We significantly reduced the number of plausible mechanisms by: 1) Performing phase plane and bifurcation analysis of the generic properties of these models and relating these properties to the results of adoptive transfers; 2) Fitting the models to the results of *in vitro* immunosuppression assays, based on inhibition of thymidine-incorporation. However, because several parameters are unconstrained, the candidate solutions are still very degenerate. In order to further narrow down the hypothesis, we optimized a proliferation assay in which the number of cycling cells as well as the number of rounds of division are quantified, by the decrease in fluorescent intensity of CFSE-stained cells measured by FACS. We developed algorithms for analysis of the histograms of CFSE-stained cells that allowed us to estimate the number of naïve and regulatory cells conjugated with APCs, as well as the maximal and effective proliferation rates of the populations of these cells. Using this new approach we were able to further discriminate between competing models for linked suppression. We conclude that linked suppression is based on a mechanism in which regulatory cells prevent the proliferation of naïve cells and the proliferation of regulatory cells is dependent on naïve cells. The molecular basis for such mechanisms is discussed.

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Control of T-cell Expansion by Regulatory CD4+CD25+ Cells

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Inflammatory Bowel Disease can be induced in lymphocyte deficient animals by adoptive transfer of naïve CD4 cells isolated from a normal animal. Emergence of the disease has been associated with colonization of the intestine by Helicobacter. In such a system, co-transfer of CD4+CD25+ cells prevents the deleterious inflammation.

In order to further characterize the function of regulatory CD4+CD25+ cells we investigated their competence in controlling other CD4-mediated acute inflammations. We followed lymphocyte deficient animals (Rag-/-) infected to various degree with *Pneumocystis carinii* (PC) that presented a typical state of chronic inflammation in the lung tissues. Analyses of those mice after adoptive transfer of CD4 subpopulations in what concern, health status, survival, tissue damage and cellularity will be presented.

Taken together our results support the idea that CD4+CD25+ regulatory cells control deleterious lymphoproliferation and inflammation. In addition, they indicate that ongoing inflammation is targeting deregulated CD4 cells expansion. Moreover, we show that co-localization and co-expansion of naïve and regulatory cells follow rules established by local environment. This study provide new insights on the mechanism that leads to tissue specific breakdown of tolerance, which will be discussed.

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T-cell Regulation During the Induction Phase of the Immunoresponse Against Self-antigens

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Non-deletional mechanisms contribute to maintaining peripheral self-tolerance. This model of dominant tolerance builds upon the persistence of immunocompetent autoreactive cells in healthy individuals, controlled by regulatory cells. It has been shown that transient depletion of T cells with different phenotypes promotes the outbreak of several organ-specific autoimmune diseases, targeted to particular tissues depending on the genetic background. However, factors defining the specificity of this autoimmune attack remain unknown.

It is well established that antigenic manipulations that enhance the immunogenicity of self-antigens lead to the development of experimental autoimmune diseases. Animals spontaneously recover and become specifically resistant to new episodes of the same disease. Some experimental evidence supports a role for T cells in resistance to a second induction of the disease. However, despite persistent efforts, it has not been possible to demonstrate *in vivo* the involvement of peripheral regulation in the induction phase of antigen specific immunoresponses.

Using different animal models we have addressed two aspects of this problem. First, the contribution of distinct T-cell subpopulations to the regulation of the induction phase of the immunoresponse against specific self-antigens. Second, to evaluate whether peripheral expansion of lymphocytes after a transient immunosuppression is driven by antigen and whether it is possible to replenish the "lymphoid space" skewed towards selected specificities.

For this purpose, we studied the effect of transient depletion of CD4+, CD8+ and CD25+ T cells in the induction phase of the immunoresponse against epidermal growth factor (EGF) and myelin oligodendrocyte glycoprotein (MOG)35-55 peptide in BALB/c and C57BL/6J mice, respectively.

We found that during the induction phase of the immunoresponse:

- T-cell depletion enhances in a dose-dependent manner the immunoresponse against the immunized self-antigen and may strengthen the specific autoimmune disease development.
- Antigen presentation also contributes to this effect.
- Induced autoimmune responses against some antigens may modulate in parallel the response against other self-antigens, in a non-random fashion.
- This effect is associated with a modulation of the regenerated repertoire against the immunized antigen.
- The regulation may involve different T-cell subpopulations with variable levels of contribution of CD4+, CD8+ and CD25+ T cells, depending on the self-antigen.

These studies fit into the model of dominant tolerance and provide evidence for the effective manipulation of natural autoreactivity to enhance specific immune responses against selected self-antigens. Autoimmunity is emerging as a paradigm for tumor immunity; however, overcoming the state of immune tolerance or ignorance of tumor antigens remains a major obstacle. We are currently evaluating the implications of our findings for cancer therapy.

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