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The works summarized in this publication were presented by their authors at a Workshop held on 24th to 26th April 1989 at the Fundación Juan March.

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Fundación Juan March

246 Workshop on Tolerance: Mechanisms and Implications

SERIE UNIVERSITARIA

Fundación Juan March

Workshop on Tolerance: Mechanisms and Implications

organized by
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Depósito legal: M-21.545/1989
I.S.B.N.: 84-7075-393-2
Impresión: Ediciones Peninsular. Tomelloso, 37. 28026 Madrid.

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Ronald H. Schwartz

INTRODUCTION

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Howard Hughes Medical Institute, Denver
(USA)

INTRODUCTION

It has long been clear that one of the critical features of the immune system in higher vertebrates is its ability to distinguish between self and foreign materials. Without such discrimination there would be anarchy and rapid destruction of the host. Therefore as the immune system evolved mechanisms designed to prevent recognition of self must have developed at the same time.

Mice and mankind appear to use 3 different clonally variable methods for recognition of foreign materials. These are immunoglobulin molecules on the surfaces of, or produced by B cells, and two different kinds of receptors on T cells, made up of $\alpha\beta$ or $\gamma\delta$ polypeptides. Theoretically it is possible that tolerance is established in a different way for each of these methods. Alternatively similar mechanisms for tolerance induction may be used by B cells and T cells. It is also possible that since at least 2 of the methods are somewhat interdependent, and B cells need T cell help in order to respond, tolerance in the T cell compartment may automatically lead to lack of response to self by B cells.

Over the years many investigators have suggested mechanisms for tolerance induction. In general such mechanisms

break down into 3 categories, clonal elimination, clonal anergy and suppression. Theories invoking clonal elimination, first suggested by Lederberg in the 1950s, depend upon the fact that T or B cells develop in a sea of self antigens, but, in an uninfected individual, in the absence of foreign material. It is suggested that the developing lymphocyte goes through a stage when engagement of its immunoglobulin or $\alpha\beta$ receptor triggers cell death. Later, developing cells become mature, and at this stage engagement of their antigen receptors is a stimulatory event. The constant presence of self antigens, and only intermittent presence of foreign antigens should allow cells specific for foreign materials to mature, but eliminate self-reactive lymphocytes.

Theories invoking clonal anergy as a mechanism for self tolerance are very similar to those which suggest clonal elimination, with the exception that they suggest that engagement of antigen receptors at a particular stage of lymphocyte development, or under particular circumstances, causes anergy in lymphocytes rather than death.

Finally under some circumstances self tolerance may be maintained by suppressor cells, which interact with antigen, or antigen receptors on other lymphocytes to reduce, or oblate completely, responses to some self components.

Recently significant advances have been made in our understanding of self tolerance, largely because a number of new techniques have been developed which allow a better examination of the problem. These new techniques include the methods of molecular biology, production of transgenic animals, development of new antibodies and improved tissue culture techniques and cell lines. The time was therefore ripe for a reexamination of the problems of tolerance, and under the generous auspices of the Fundacion Juan March a workshop was organised in April 1989 in Madrid to discuss the new data. The workshop was divided into 2 sections, a meeting of about 12 foreign guests and 25 Spanish experts in the field which went on for 2 days, and a set of public seminars by some of the participants which was given on the third day.

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CONTROL OF T CELL DEVELOPMENT BY THE α β T CELL RECEPTOR FOR ANTIGEN

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T cell receptor (TCR) gene segments begin to rearrange in CD4-8- thymic lymphoblasts. In scid mice the development of T cells is arrested at this early stage as the scid thymus does not contain any $CD4^+$ or $CD8^+$ lymphocytes.

This block in T cell development can be overcome by introducing productively rearranged TCR genes into the scid strain which results in the formation of $CD4^+8^+$ lymphocytes.

While this early differentiation step requires TCR's of any specificity, later developmental stages depend on the specificity of the TCR: in scid mice, a transgenic TCR restricted by D^b class I MHC antigens allows the formation of $CD4^-8^+$ but not $CD4^+8^-$ lymphocytes in D^b positive but not D^b negative animals. Thus, a TCR-MHC interaction in the absence of nominal antigen is required for the generation of mature T cells, and this interaction determines the CD4/CD8 phenotype.

If both nominal antigen and presenting MHC antigen are present developing T cells are deleted at an immature $CD4^{+}8^{+}$ stage preventing the formation of more mature and functional autoaggressive T cell progeny.

These experiments indicate that the immune system first learns about self by positive and negative selection of self recognizing lymphocytes from a continuously turning over pool of lymphocytes without requirement for idiotypic network interactions.

T CELL TOLERANCE AND SELECTION

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Positive selection and deletion of αβ T cell receptor-bearing T cells in the thymus must involve the αβ receptor itself. Apparently at one time engagement of the αβ receptor can lead to positive selection and at another, the same event can lead to elimination of the cell bearing it. Several hypotheses have been advanced to account for this dichotomy. We have recently shown that engagement of αβ receptors can cause the disappearance of all medullary, mature αβ-bearing T cells, and about half the cortical, immature αβ-bearing thymocytes. This seems to be due to death of cortical thymocytes, caused be Ca^{++} fluxes induced by triggering via their αβ receptors. Some $\alpha\beta^{+}$ immature thymocytes do not flux Ca^{++} upon engagement of their receptors, these cells are resistant to receptor-mediated clonal deletion and may, therefore, be the targets of receptor-mediated positive selection.

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T-CELL PRECURSORS: IN VITRO DIFFERENTIATION & ACQUISITION OF EFFECTOR FUNCTIONS

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T-cell precursors arising from hematopoietic stem cells colonize the thymus during ontogeny, where they undergo a complex maturational process involving genotypic and phenotypic changes in the expression of distinct surface molecules. Later, they migrate to the periphery as immunocompetent T cells expressing clonally-distributed T-cell receptor (TCR) structures. Four different TCR genes (α, β, γ and δ) have thus far been identified and shown to be specifically rearranged and expressed throughout intrathymic T-cell development. They code for two distinct types of heterodimeric TCR: the common MHC-restricted α/β heterodimer expressed on most functional T lymphocytes, and the recently described γ/δ TCR complex, expressed on a minor T-cell subset. Both structures are expressed in association with the monomorphic CD3 (T3) complex, but they seem to be acquired independently by distinct intrathymic subpopulations.

Developmental studies in mice support that the γ/δ TCR appears first in ontogeny on early "double negative" ($CD4^-$ $CD8^-$ thymocytes. Further maturation leads to a gradual decrease of γ/δ-bearing cells. In contrast, α/β TCR expression increases throughout T-cell ontogeny concomitantly with the

acquisition of CD4 and/or CD8 molecules by mature T cells, expression of γ/δ TCR being restricted to a small population of $CD4^-$ $CD8^-$ adult thymocytes and peripheral T cells. These findings suggest that γ/δ-bearing $CD4^-CD8^-$ cells may define a separate T-cell lineage whose intrathymic development precedes that of classical α/β mature T cells. Nonetheless, the presence of γ-gene rearrangements in mature α/β-bearing T cells, as well as the finding of partial β-gene rearrangements in γ/δ TCR^+ cells, indicate that both T-cell lineages may derive from a common precursor. At the present, however, the regulatory mechanisms underlying these developmental processes remain poorly understood, and precursor-product relatinships involving the various intrathymic subpopulations continue to be disputed, making it difficult to establish direct correlations between the described patterns of TCR gene expression and a functional pathway of T-cell development.

"In vitro" differentiation approaches were used to analyse the precursor potential and the putative progeny of a minor population of adult human thymocytes which lack conventional T-cell markers ($CD2^-1^-3^-4^-8^-$, i.e.$T11^-6^-3^-4^-8^-$) but express CD45 (i.e.T200) and CD7 molecules, suggesting that they are the most immature intrathymic progenitors. Moreover, only γ-chain functional RNA messages are expressed in this subset, whereas α- and β-chain TCR genes remain in germ-line

configuration. Interestingly enough, "in vitro" culture of this subpopulation in the presence of interleukin 2 (IL-2) led to an extensive cellular proliferation and the concomitant differentiation into both γ/δ and α/β TCR^+ thymic subsets. These data support the involvement of the IL-2 pathway in the intrathymic maturation of early T-cell precursors. Furthermore, they provide a useful "in vitro" system to induce expression of α/β as well as γ/δ TCR structures in developing thymocytes, making it feasible to investigate the cellular and molecular basis of T-cell repertoire selection and development operating in T-cell differentiation.

TOLERANCE INDUCTION IN THE ADULT UNDER COVER OF MONOCLONAL ANTIBODIES TO T-CELL ADHESION MOLECULES

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The introduction of foreign proteins, bone marrow or tissue grafts into immunologically mature animals usually results in vigorous immune responses.

We have found that monoclonal antibodies (Mabs) to CD4, CD8 and LFA-1 injected in-vivo create a tolerance permissive environment for all 3 categories of antigen.

Tolerance does not require that the antibodies necessarily ablate mature T-cells because antibody fragments or non depleting isotypes are also effective.

Bone marrow grafts can be administered under antibody cover to establish transplantation tolerance to tissue grafts.

For genetic differences across multiple minors or class I $^{+}$ minors antibody therapy is sufficient.

For complete MHC + minor mismatches tolerance requires the addition of sublethal doses of irradiation.

Non-depleting Mab regimes can be used to establish transplantation tolerance to multiple minor mismatched skin grafts, and short courses of appropriate combinations of CD4 and CB8 mabs permit long-term acceptance (>120 days) of totally mismatched skin grafts.

It is proposed that therapeutic interventions which isolate antigen-binding T-cells from each other, and therefore from collaboration, render them tolerance susceptible.

INDUCTION OF TOLERANCE BY EMBRYONIC THYMIC EPITHELIAL GRAFTS IN BIRDS AND MAMMALS

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In situ implantation of a quail wing bud into a chick embryo at 4 days on incubation (E4) regularly results in the normal development of the implant followed by its acute rejection starting within two weeks posthatching. If the epithelial thymic rudiments of the quail donor are implanted into the branchial arch area of the chick recipient after partial removal of its own thymic primordia, a chimeric thymus develops in the chick host and this induces tolerance to the quail wing by the chick recipient. The species identity of cells in chimeric thymuses was mapped using Feulgen-Rossenbeck' staining and immunolabelling with monoclonal antibodies directed against quail or chick B-L antigens. Certain lobes contained only chick cells both at the stromal and hemopoietic cell levels. Others had a quail epithelial stroma containing host hemopoietically derived cells. Only chimeras in which at least one third of the thymic lobes were chimeric showed permanent tolerance to the grafted wing.

Similar results have been obtained when the rudiment of the bursa of Fabricius (not yet colonized by hemopoietic cells), instead of the limb, has been substituted at E5 for the chick host bursa.The quail bursa is rejected from about two weeks after birth in the absence of thymic grafts while it is maintained for at least three months if thymic epithelium from the same donor is implanted. After

that time, the bursa undergoes its normal physiological involution.

In mammals a comparable experimental paradigm has been devised by using BALB/C Nude mice as host onto which the third branchial pouch areas of E10 embryos of the C3H strain are implanted subcutaneously at birth. Thymic tissue develops from the graft in which the hemopoietically derived cells (lymphocytes and medullary dendritic cells and macrophages) are all of host origin (H2d type) while the thymic epithelium is derived from the graft (H2k type). Such T cell reconstituted mice when challenged with skin grafts at 3 months of age reject C57B1/ 6(H2b) - third party grafts but tolerate both normal BALB/C and C3H skins.

Altogether these results indicate that thymic epithelium is able to present self antigens to differentiating T cells in a tolerogenic form.

THE THYMUS AND T CELL TOLERANCE

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T cell differentiation in the thymus is controlled by MHC molecules and involves a complex process of positive and negative selection: positive selection generates T cells with specificity for self (thymic) MHC molecules whereas negative selection deletes a portion of these cells, i.e. T cells with overt auto-MHC reactivity. Although the identity of the cell types that control thymic selection is still controversial, the bulk of evidence suggests that positive selection reflects T cell contact with MHC molecules expressed on cortical epithelial cells whereas negative selection is controlled largely though not exclusively by intrathymic bone-marrow-derived cells. Evidence that thymic epithelial cells might contribute to negative selection has come from studies with parent a T cells generated in a - (a x b) F_1 chimeras prepared with supralethal irradiation (sufficient to destroy all detectable host-type APC). In the case of Ia-restricted $CD4^+$ cells, donor-derived T cells differentiating in the endogenous thymus of the host or in a host-type thymus graft show considerable tolerance to host-type Ia molecules. Tolerance is associated with deletion of T cells expressing

host-reactive TCR molecules. Tolerance of $CD4^+$ cells is induced intrathymically, presumably through contact with host Ia molecules expressed on a non-marrow-derived component of the thymus (? epithelial cells). No such tolerance occurs when parent *a* CD_4^+ cells differentiate in thymectomized irradiated (*a* x *b*) F_1 hosts given a parent *a* thymus graft. This latter finding suggests that post-thymic exposure of $CD4^+$ cells to host Ia molecule fails to lead to tolerance induction.

FAILSAFE MECHANISMS OF T CELL TOLERANCE

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Although deletion in the thymus is certainly an effective way of inducing self tolerance, it cannot be the only mechanism. There must be backup systems to ensure the maintenance of tolerance should deletion be imperfect. We have found two sorts of peripheral fail safe mechanisms able to induce tolerance in mature cytotoxic T cells *in vivo*.

The first is a form of starvation. Cytotoxic T cells specific for the alloantigen Qa-1 require T help at the time of activation. In the presence of activated helper T cells they respond. In the absence of help they become irreversably unresponsive, remaining that way for periods up to seven months.

The second is some form of suppression. Chimaeric mice which carry two different thymuses are tolerant of all the relevant antigens, yet each T cell ought to be tolerant only of the antigens presented by the thymus in which it matured. This form of tolerance is not set up instantaneously. Several mice began by responding to the tolerogen and then later became unresponsive. In addition, the unresponsiveness extends to include control antigens which

are presented on the same cell as the tolerizing antigens. In this respect, this form of peripheral tolerance differs from the first which is specific only for the tolerizing Qa-1 antigen.

We are now testing whether the unresponsiveness in either system is transferable and whether we can find a means of reversing it.

THE ROLE OF THE COSTIMULATORY SIGNAL IN DETERMINING THE OUTCOME OF T_H1 ANTIGEN RECEPTOR OCCUPANCY

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Antigen stimulation of $CD4^+T_H1$ clones with either antigen-presenting cells chemically modified with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI) or purified Ia molecules in planar membranes induces a state of hyporesponsiveness to subsequent stimulation with normal presenting cells and antigen as measured by both thymidine incorporation and interleukin-2 production. The hyporesponsive state lasts for more than one week, although the cells remain viable as manifested by their ability to respond to exogenous interleukin-2. Induction of unresponsiveness requires new protein synthesis and is accompanied by the production of IL-3, IFN-γ, increases in TCRβ mRNA, and small increases in IL-2 receptor expression; however, little IL-2 is produced. The critical biochemical event for the induction of nonresponsiveness is a rise in intracellular calcium. Entry into the hyporesponsive state is blocked by EGTA or cyclosporine and the state can be chemically induced by the calcium ionophore, ionomycin. Addition of allogeneic accessory cells during the exposure to ECDI-treated APC and antigen blocks the induction of nonresponsiveness and induces a proliferative response from the T cell clone. These effects could not be mimicked by addition of a phorbol ester (PMA) or soluble lymphokines and addition of the allogeneic cells does not

increase hydrolysis of phosphatidylinositol polyphosphates, nor does it activate protein kinase C as measured by phosphorylation of the T cell receptor gamma chain. These observations suggest that occupancy of the antigen-specific receptor on $CD4^+$ T_H1 clones, in the absence of a costimulatory signal, leads to an increase in intracellular calcium, activating a biochemical program that eventually prevents the cell from producing IL-2 in response to normal activation signals. If, however, an accessory cell costimulatory signal is present, the T cell clone divides and nonresponsiveness is prevented. The costimulatory signal does not appear to be transduced by activation of protein kinase C, suggesting that three biochemical signals are required for activation of normal T cell clones. These experiments appear to be at odds with the ability of either PMA and ionomycin or anti-CD3 monoclonal antibodies to induce a T cell proliferative response. Such responses, however, can only be obtained at high cell density. T cells plated at limiting dilution do not respond to PMA and ionomycin, although they do respond to IL-2. An analysis of log cell number versus log response curves revealed a slope of 2 for both PMA and ionomycin and anti-CD3 antibody stimulation, which could be converted to a slope of 1 with the addition of accessory cells. These results suggest that the stimulation of T cell clones with these reagents at high density is the consequence of contaminating antigen-presen-

ting cells or the ability of highly activated T cells to provide costimulatory signals to each other.

THE ROLE OF CLONAL SELECTION AND SOMATIC MUTATION IN AUTOIMMUNITY

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Antibodies to DNA (anti-DNA) are prominent auto-antibodies in the sera of patients with systemic lupus erythematosus (SLE) and of MRL/M_p -lpr/lpr (MRL/lpr) mice, a murine model of SLE. The levels of these auto-antibodies have diagnostic and prognostic significance. Moreover, a direct role of anti-DNA in disease pathology has been established by correlation of antibody levels with disease activity and identification of anti-DNA at sites of tissue injury. Two competing models to explain anti-DNA have emerged. The first suggests that anti-DNA result from polyclonal B-lymphocyte activation and are the consequence of antigen-nonspecific disturbances of B and/or T cells. The extensive diversity of anti-DNA has been interpreted as evidence for the polyclonal activation model. In addition, reports of idiotypes expressed on a large fraction of anti-DNA suggest that anti-DNA are encoded by germline variable (v) - region genes. The second model proposes that antigen stimulates anti-DNA production. Although nonspecific immune disturbances may exist in SLE patients, in this model their role is to allow or promote induction of autoantibodies by antigen. It is reasonable to postulate DNA as the inciting antigen, although a non-DNA self of foreign antigen could fulfill this role. To provide further insight into the etiology

and structure of anti-DNA, we have investigated the specificity and primary structure of monoclonal anti-DNA obtained from spleen cells of autoimmune MRL/lpr mice. The goal is to determine the genetic basis of anti-DNA diversity and thereby to distinguish these models of anti-DNA production.

BEYOND CLONAL SELECTION AND NETWORK

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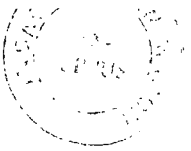
The rapid progress in the genetic, molecular and cellular characterization of the immune system has had little impact on clinical problems. We continue to treat allergy as before IgE was known, we have no specific treatment for autoimmune diseases, we are unable to tolerize the recipient of an organ to the tissues of the donor, and we seem incompetent to derive effective vaccines protecting from parasite infections the larger part of the world population. To tackle these problems, it seems to me, modern immunology will have to make the step from molecular and cellular biology (component analysis and local rules) to systemic biology (approaches to the global behaviour of an organized system). Immune properties such as learning, memory and self-nonself discrimination, are likely to be "distributed", and thus result not only from the presence or activity of a given set of components, but from their interactions and dynamic organization.

The theory of idiotypic networks constitutes a frame in which systemic immunology could develop. Unfortunately, ever since its proposition, the idiotypic "network" has been essentially considered, both theoretically and empirically, in the context of immune responses and their regulation, leaving aside the real novelties in the idea: functional autonomy, global behaviours and positive definition of self. We have been concerned, over the last few years in



exploring these aspects, first of all that of functional autonomy. We found that normal mice, even if secluded from all environmental stimulation (antigen-free) display considerable levels of mature lymphoid activities. Thus, 10 to 20% of all splenic B, CD4 and CD8 T cells in these mice are activated blasts, many of which are engaged in mitotic cycle and perform effector functions, such as antibody secretion, and help or suppression of B lymphocytes. These observations falsify one of the tenets of the clonal selection theory, but they also limit a putative functional network to a minority of the lymphocytes in the adult immune system.

The analysis of reactivities in the compartment of activated lymphocytes in neonatal and adult mice has revealed some striking features. Perinatal antibody repertoires are encoded by germ-line genes of the most D-proximal VH homology families, with little or none random diversity introduced in the form of N-sequences. Most importantly, such antibodies very frequently react with other members of the set, embodying a highly connected network of V-regions, while reactivities with "somatic" self components are not particularly overrepresented. In contrast, activated B cell repertoires of the adult show a very marked bias for autoreactivities, which is learned by positive selection, as shown by the time course analysis over the first months of

life. Adult natural antibodies, in addition, show no particular preference in VH-gene utilization and "conventional" N-diversity. Repertoire analysis of bone-marrow and peripheral resting B cells in adults reveals the rapid elimination of emergent cells expressing D-proximal VH-genes from this compartment, that is accompannied by a deletion of auto-reactive specificities among immunocompetent resting B cells. Furthermore, antibodies isolated from this set show low levels of idiotypic connectivity. We interprete these observations by the recruitment of emergent clones with self affinities (be them idiotypic or to components of the somatic self) into the activated cell compartment, and/or by paralysis and rapid decay brought about by supra-optimal levels of ligand binding.

Evidence has been produced for the T cell-dependence of the positive selection of autoreactive B cells. The lack of pathogenic effects of such physiological autoimmunity should be explained, therefore, on other grounds. Clonal stimulation in vivo of antibody reactivities in the autonomously activated compartment has revealed that no immune responses can be obtained, in contrast to the rapid and high titred responses generated by activation of equivalent clones in the disconnected lymphocyte set. Furthermore, the analysis of lymphocyte population dynamics reveals that the large cell compartment of normal animals is maintained with little cell

division, and the study of serum natural antibody concentrations over time shows dynamic patterns that are very different from immune response dynamics: individually characteristic fluctuations within rather narrow ranges, following oscilatory or chaotic attractors. These observations could explain why normal autoantibodies do not undergo somatic mutation and further antigen-dependent selection, as well as the fact that such autoreactivities are trully at equilibrium with the somatic self.

The evidence above, together with a number of theoretical considerations, lead to a model considering the immune system of mammals composed of two separable domains: the central immune system (CIS), composed of activated lymphocytes with autonomous functional behaviours, embodying self-related repertoires and a network of high connectivity; the peripheral immune system (PIS), including the large majority of mature lymphocytes, which are disconnected, functionally at least, from the internal environment of "somatic" self and V-regions of the CIS. The former is organized as a network, ontogenically established, and recursively selects its components by recruiting newly araising specificities into the activated compartment, on the basis of metadynamic rules that include the "learning" of the somatic self; we believe that its biological importance is related

to "self assertion", that is, the establishment of the molecular identity of the individual and tolerance by positive definition of self. In contrast, the PIS consists of isolated clones with no network organization, devoid of connectivity with molecular self; their role in normal physiology is the development of specific immune responses to external antigens, characterized by rapid and large clonal amplifications, in a typical stimulus-response allonomous behaviour, essentially regulated by antigen alone.

These perspectives offer new insights to the questions of pathological autoimmunity and the establishment of peripheral, post-thymic tolerance, particularly, the acquired tolerance to transplantation antigens. It is our conviction that the possibility of analysing the physiology of auto-immunity, so far unacceptable in conventional theory, will contribute to a better understanding of its pathological disturbances. Concrete prospects are that autoimmune pathology will be related to disturbances in functional connectivity, such that a possible therapeutic approach would be the reinforcement of the CIS, aiming at connecting the pathologically "disconnected" autoreactive clones. The recent therapeutic success of the injection of large amounts of normal immunoglobulin in a variety of autoimmune diseases would support this contention. Conversely, the analysis of clonal repertoires

in mice that were neonatally tolerized to allogeneic tissue grafts has revealed that tolerance occurs in the absence of clonal deletion and correlates with "natural activation" of the specific alloreactive cells. It would be possible, therefore, to consider specific manipulations of adult individuals, aiming at recruiting into the CIS from the PIS, the appropriate alloreactive lymphocytes before organ transplantation.

The most exciting possibility open by the present views is, however, the whole area that could be designated as "immunosomatics". Given that physiological tolerance does not represent ignorance of the molecular somatic self, but rather, the dynamic equilibrium between self and the immune network components, it would appear that manipulating the CIS could provide corrections or compensations to malfunctions in other biological systems, even if they would not have an immunological ethiopathogeny.

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B CELL TOLERANCE IN LYSOZYME/ANTI-LYSOZYME TRANSGENIC MICE

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Efforts to understand the mechanism of self-tolerance focus on two central issues. Firstly, how do variables such as the time and site of antigen encounter or the presence or absence of "costimuli" influence tolerogenicity versus immunogenicity of autologous antigens? Secondly, what cellular mechanisms keep potentially self-reactive cells silent? Given the diversity of self-antigens which must be tolerated, and the diversity of lymphocytes which must be tolerized, it seems likely that different solutions will be used in different situations. Ideally, we need to be able to manipulate both the pattern of expression of self-antigens, and the frequency and receptor status of self-reactive cells. Transgenic mice provide the genetic tools with which to achieve these manipulations.

We have used hen egg lysozyme (HEL) as a model self-antigen by generating multiple lines of HEL-transgenic mice, and focussed on the fate of self-reactive B-cells by mating HEL-transgenic mice with anti-HEL immunoglobulin transgenic mice. In the resulting "double-transgenic" offspring,

tolerance to HEL is maintained by a mechanism which efficiently silences HEL-binding B-cells but does not result in clonal deletion of these B-cells. The silencing phenomenon is intimately linked to a dramatic downregulation of membrane IgM but not IgD on the B-cells, is capable of acting on mature B-cells, is dependent on the concentration of HEL in the mice, and is reversible when the B-cells are removed from constant stimulation by HEL. These features of the silencing mechanism seem well suited to maintaining self-tolerance in the B-cell repertoire, where hypermutation of receptors in mature B-cells poses a unique regulatory problem.

B LYMPHOMA MODELS FOR ANTIGEN-INDUCED CLONAL ANERGY OF IMMATURE B CELLS

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A number of B lymphoma-derived cell lines arrest their growth upon treatment with anti-immunoglobulin, used as a surrogate for antigen. Of these, the most intensively studied is WEHI-231. The cell surface phenotype of these cells is similar to that of normal immature B cells. The growth of these cells is inhibited by over 90% within 24 hr. after the addition of anti-Ig, and completely soon thereafter. This growth inhibition is overridden by the presence of lymphokines derived from activated helper T cells or by the presence of polyclonal B cell activators such as bacterial lipopolysaccharide (LPS). Similarly, antigen-induced clonal anergy or deletion of normal immature B cells fails to occur in the presence of LPS or carrier-primed helper T cells. Thus, by a number of criteria, the anti-Ig-induced growth inhibition of WEHI-231 cells is similar to the clonal anergy or deletion response of immature B cells to antigen.

We have demonstrated that anti-Ig triggers the phosphoinositide signal transduction pathway in WEHI-231 cells, as is also true in mature splenic B cells. Anti-Ig stimulates phospholipase C to hydrolyze PIP_2, generating diacylglycerol, which activates protein kinase C, and inositol triphosphate, which induces elevation of intracellular calcium, a well-established second messenger. For most receptors, a given receptor always induces generation of the same

second messengers, regardless of the cell type in which it is found or the biological response that is generated. Different responses can reflect different downstream events triggered by the same second messengers in different cells. Therefore, it is possible that membrane Ig (mIg) signals via PIP_2 hydrolysis in both mature B cells, where antigen triggers growth promoting responses, and in immature B cells, where antigen induces clonal anergy or deletion. Nonetheless, the demonstration that mIg induces phosphoinositide signaling does not necessarily mean that this is the only signaling mechanism of mIg, or even that it is the signaling reaction that mediates the biological response. We have utilized phorbol esters (which activate protein kinase C like diacylglycerol) and calcium ionophores to see whether mimicking phosphoinositide second messengers will result in growth inhibition. Reasonable doses of these two pharmacological agents do induce growth inhibition when used in combination. By a number of criteria, the growth inhibition induced by the combination of phorbol ester and calcium ionophore is similar to the growth inhibition induced by anti-Ig. For example, in each case the cells arrest in G_1 phase of the cell cycle and LPS overcomes the growth inhibition. The growth inhibition induced by phorbol ester + ionophore is, however, slower and less efficient, suggesting that a third intracellular mediator may be involved in addition to diacylglycerol and calcium.

An advantage of cell lines such as WEHI-231 is the possibility of isolating mutants with altered properties. We have isolated over 30 independent mutants of WEHI-231 that fail to arrest growth in response to anti-Ig. Some ot these mutants fail to make normal amounts of mIgM. More interesting mutants have normal membrane IgM, but are defective in either signal transduction or response to second messengers. One of these mutants appears to be defective in phospholipase C, the enzyme that hydrolyzes PIP_2 upon receptor stimulation. A second mutant has normal generation of phosphoinositide second messengers but does not exhibit growth arrest in response to either anti-Ig or to phorbol ester + ionophore. This mutant suggests that the diacylglycerol and/or calcium second messenger pathways are required for the growth arrest in response to anti-Ig. Several other mutants appear to be defective in the continued elevation of intracellular calcium, although immediate elevation of intracellular calcium is normal. Thus, the phenotypes of these mutants support the idea that membrane Ig-stimulated phosphoinositide breakdown mediates growth inhibition in this cell line. Further experiments will be required to determine whether antigen-induced clonal anergy of immature B cells is also mediated by this signal transduction pathway.

THE T CELL REPERTOIRE. THE IMPACT OF SELF AND THE ENVIRONMENT

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The products of some alleles of some mouse genes (eg. Mls) react with all mouse T cells bearing a particular V_β as part of their $\alpha\beta$ T cell receptors. Consequently, T cells bearing these target V_β's are deleted in mice expressing these so-called super antigens. This is not an artifact peculiar to laboratory mice. Deletions of this type are common in wild mice too. Super antigens with similar properties are also produced by microorganisms and include a group of toxins produced by staphylococcus aurens. Some of the pathogenic properties of these toxins may be the consequence of T cell stimulation. Super antigens may exist in mice to delete the T cell targets of some bacterial toxins and thereby protect the host animal against toxic attack.

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SUMMARY

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SUMMARY

The workshop was organised to allow separate discussion of tolerance of $\alpha\beta$ T cells and B cells. Since so very little is known at the moment about the specificity and function of $\gamma\delta$ T cells, tolerance of this type of lymphocyte was not discussed.

T cell tolerance to antigens which are expressed in the thymus is caused by clonal deletion of potentially reactive thymocytes at a particular maturational stage. This deletion is presumably caused by engagement of the receptors on developing T cells with antigen. Several different types of experiments have documented this phenomenon, some involve anti-Vβ or anti-$\alpha\beta$ antibodies, others, transgenic mice. Experiments described at this workshop suggested that the stage at which thymocytes become sensitive to such deletion can be quite tightly defined, it appears to be at some time after the maturing cell has expressed receptors on its surface, but before the cell has become fully functional.

With the idea that T cell tolerance can be caused by clonal deletion firmly in mind, the question arose whether this is the only mechanism that causes nonresponsiveness in these cells, or whether perhaps evolution has designed another,

failsafe mechanism, to deal with self components which are not well represented in the thymus or which appear late in development, for example at adolescence. Several presentations at the workshop suggested alternate methods for the establishment of T cell nonresponsiveness. For example, T cells in chickens or mice can be rendered nonresponsive to tissue grafts carrying antigens which should not reach the thymus. Although not completely established, it is possible that this type of nonresponsiveness is caused by clonal anergy rather than deletion, as experiments in tissue culture and in animals have shown. Most exciting of all is the possibility, raised at this workshop, that such anergy can be deliberately induced by appropriate treatment of the host. Were such protocols to become available they would have tremendous implications for transplantation and treatment of autoimmune disease.

B cell tolerance appears to be an entirely different matter. Old and new experiments have indicated that complete tolerance to self on the part of B cells does not exist, nor is it to be expected. B cells do seem to be monitored for self reactivity, at least in part, however. This was best illustrated at the workshop by experiments involving transgenic mice, expressing antibody against self. In these animals B cell nonresponsiveness appeared to be controlled by clonal anergy. Examination of antibodies produced in autoimmunity supports

in part this point of view, most self reactive B cells appear to be silenced with only a few slipping through the screening process of tolerance to produce autoantibodies. Why and how some B cells are not inactivated was a debated question, and the idea was raised that anergy is usually maintained through an antibody network, through aberrations of which B cells may sometimes escape.

Overall the workshop succeeded well its purpose. The issues and different mechanisms of tolerance were all discussed, and in some areas unexpected concensus was reached. Much remains to be done, but the prospects for a theoretical understanding of the matter, coupled with the many potential therapeutic applications of such understanding will lead to a good deal of vigorous research on the matter in the future, in Spain and elsewhere.

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