

Immunologic tolerance for immune system-mediated diseases

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Induction of long-term, antigen-specific immunologic unresponsiveness holds great promise for the treatment of many immune system-mediated diseases, including asthma, allergies, autoimmune diseases, and transplant rejection. Unlike current immunosuppressive treatments, immunologic tolerance therapies would affect only the undesired immune responses, leaving protective immunity intact. A variety of approaches to immunologic tolerance induction are being taken, reflecting the molecular and cellular complexity of immune system activation and regulation. The presentations summarized in this report represent promising strategies, some of which are being evaluated in advanced animal models and human clinical trials. Approaches presented include the following: interference with costimulatory signals in T-cell induction, T-cell receptor antagonism by altered peptides, exploitation of antigen-induced apoptosis to eliminate undesired T cells, opposition of inflammation by the induction of regulatory cytokines, induction of transplant tolerance by mixed chimerism, and deviation from deleterious allergic antibody responses by use of immunostimulatory DNA sequences. These multifaceted approaches are strongly supported by knowledge of basic immune mechanisms, which should facilitate the rational development of these therapies for controlling immune-mediated diseases. (*J Allergy Clin Immunol* 1999;103:362-70.)

Key words: Allergy, anergy, apoptosis, asthma, autoimmune disease, costimulatory molecules, cytokines, immunostimulatory DNA sequences, tolerance, transplantation

Our understanding of the pathways and molecules involved in the initiation and regulation of immune responses has advanced tremendously in the last several years. This knowledge has been used to develop approaches for the induction of tolerance or unresponsiveness as an intervention for immune system-mediated diseases, including asthma and allergic diseases, autoimmune diseases, and rejection of transplanted organs and tissues. The American Academy of Allergy, Asthma and Immunology and The National Institute of Allergy and Infectious Diseases jointly sponsored the symposium entitled, "Immunologic Tolerance for Immune System Mediated Diseases," which was held December 8, 1997, at the National Insti-

Abbreviations used

ALPS:	Autoimmune lymphoproliferative syndrome
APC:	Antigen-presenting cell
CTL:	Cytolytic T lymphocyte
EAE:	Experimental autoimmune encephalomyelitis
FasL:	Fas ligand
GVHD:	Graft-versus-host disease
ISS:	Immunostimulatory DNA sequences
MBP:	Myelin basic protein
MS:	Multiple sclerosis
ODN:	Oligodeoxynucleotide
OVA:	Ovalbumin
SLA:	Swine lymphocyte antigen
TCR:	T cell receptor

tutes of Health. The program was planned by Howard B. Dickler, MD (Bethesda), and Philip Fireman, MD (Pittsburgh). Eight outstanding presentations on research advances in this area were provided by leading scientists who focused on both the basic knowledge gained and the promising clinical applications. This report summarizes the highlights of those presentations.

INDUCTION OF IMMUNOLOGIC TOLERANCE AND UNRESPONSIVENESS: AN OVERVIEW

Dr Jeffrey Bluestone (University of Chicago) presented an overview of the induction of immunologic tolerance and unresponsiveness, with specific reference to his group's experience in preventing transplant graft rejection. The selective inactivation of unwanted immune responses in an antigen-specific manner holds the promise of controlling autoimmune diseases, allergies, and transplant rejection without the considerable toxicity and risk of blunting resistance to infections and tumors seen with the broadly immunosuppressive drugs currently in use.

Research over the past decade has shed light on the mechanisms used by the immune system to prevent lymphocytes from reacting to self-antigens. Elimination of autoreactive T cells in the thymus is the primary route, backed up by mechanisms of peripheral tolerance. Peripheral tolerance is maintained by the inactivation of lymphocytes outside the thymus by differential signaling pathways and is the mechanism that is most amenable to therapeutic strategies.

Two major signaling cascades are needed to activate T cells responding to antigen. The signal 1 cascade origi-

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nates from T-cell receptor (TCR) recognition of a specific peptide antigen in the context of the appropriate self-MHC molecule. This leads to activation of associated molecules, termed the CD3 complex, which play the key role in transducing TCR signals. However, unless the costimulatory signal 2 is provided to the T cell during TCR engagement by means of costimulatory ligands found on antigen-presenting cells (APCs), T-cell activation will be partial and abortive. For example, costimulation by the interaction of the T-cell CD28 molecule with the B7 molecule found on APCs is essential for eliciting T-cell activation. Even with optimal TCR stimulation, if CD28-B7 interaction is blocked, the T cells are rendered tolerant.

Strategies to induce tolerance by interfering with signal 1 have been successfully used by Dr Bluestone and colleagues in preclinical and clinical studies to prevent allograft rejection. One of the initial approaches used the anti-CD3 mAb OKT3 to interfere with TCR signal transduction. However, early studies showed that OKT3 caused widespread T-cell stimulation on first use, resulting in a major release of cytokines from T cells with resultant adverse effects. To eliminate this, an anti-CD3 antibody was modified to prevent its binding to cells by Fc receptors. Studies showed that this non-Fc-binding anti-CD3 antibody retained immunosuppressive activity, preventing primary and secondary skin allograft rejection in mice, without the first-dose adverse effects.¹

The modified anti-CD3 antibody also differed from the prototype OKT3 antibody in significantly altering the function of treated T cells. The antibody left intact signaling events that led to certain activation pathways, for example nuclear factor of activated T cells transcription, but blocked other downstream signaling events such that T cells were only partially triggered, resulting in unresponsiveness. Analyses of the cytokines released by T cells after treatment with the modified anti-CD3 mAb revealed that only T_{H1} -type cells were inactivated, leaving T_{H2} responses intact.² Furthermore, the inactivating effect was limited to activated T cells; therefore anti-CD3 in the presence of antigen led in effect to antigen-specific tolerance induction, even though the antibody itself was not antigen specific.

A humanized nonmitogenic anti-CD3 antibody has been used in 7 human kidney and kidney-pancreas transplant recipients. In 5 of the 7 patients, the treatment reversed acute rejection without toxicity, led to lower depletion of T cells than the broadly immunosuppressive drugs in common use, and increased the serum level of the T_{H2} cytokine IL-10, which may play a role in the treatment's effectiveness.

Recently, exciting results were obtained by strategies to block signal 2.³ The soluble molecule cytolytic T lymphocyte antigen-4 (CTLA-4)-Ig, which is capable of binding to the CD28 molecule of T cells and preventing its interaction with B7, was used to treat diabetic mice receiving allografts of islets of Langerhans. The treatment achieved indefinite maintenance of the graft and

tolerance to re-engraftment, while maintaining the ability of the mice to reject third part allografts.

Similar, but less complete, effects were obtained in a primate model. Monkeys injected with CTLA4-Ig over a 2-week period at the time of the islet transplant maintained the graft much longer than controls, but engraftment was not permanent. Additional strategies appear to be needed. However, analyses of T cells from the allografted CTLA4-Ig-treated monkeys showed that donor-specific T-cell unresponsiveness was achieved, a major aim of these preclinical studies.

Other inhibitors of costimulation are being studied. Antibodies to another important T-cell surface receptor, the CD40 ligand, prolonged islet allograft survival. Significantly, anti-CD40 ligand potentially blocked antibody responses in mice, a feature of potential importance for treating asthma and certain autoimmune diseases.

In summary, the ability to selectively block signal 1 or signal 2 in T-cell activation can be exploited to achieve peripheral tolerance. The precise mechanism by which these strategies function is not completely understood, but it is related to an imbalance in the T-cell signaling process, leading to inactivation of cells and skewing of responses. The ability to target distinct lymphocyte receptors suggests that multiple approaches can be combined to fine-tune the desired effects. Research is needed to define new potential target receptors on T cells and to design pharmacologically useful small molecule drugs. This would permit wide use of these approaches in autoimmune diseases, allergies, and transplantations in which antigen-specific tolerance is the key to control and cure.

HOW T CELLS RECOGNIZE ANTIGEN

Dr Paul Allen, (Washington University, St Louis) dealt with a key topic in immunologic tolerance, the mechanism of T-cell antigen recognition. Far from being an all-or-none effect, T cells can recognize a range of related peptides, including less than optimal ligands. This feature of T-cell antigen recognition has major implications for the development of the T-cell repertoire and tolerance induction.⁴

T cells show high specificity for very short fragments of peptide antigens presented by MHC molecules on the surfaces of APCs. The actual contact with the peptide antigen involves several key amino acid side chains that directly interact with the TCR. If this binding is optimal, the peptide is termed a full agonist, defined by its ability to trigger all T-cell functions, including proliferation, cytokine release, and complete effector activity. An MHC-binding peptide with TCR contact residues so different that the T cell cannot bind meaningfully is termed a *null ligand*, which probably constitutes the majority of peptides for a given T-cell specificity. More interesting are peptides that differ slightly from the full agonist peptide, termed *altered peptide ligands*, which still bind to the MHC but differ in their ability to be recognized by a given TCR. Some of these may elicit full T-cell functions if provided at higher concentrations, this being the defin-

ition of a weak agonist. Other peptides trigger a much less than optimal T-cell response, resulting in the stimulation of some, but not all, of the T-cell functions. For example, T cells recognizing such an altered peptide ligand may secrete cytokines but not be able to proliferate.

Perhaps most interesting are the antagonists. These altered peptide ligands specifically inhibit the response of T cells to the agonist peptide. Dr Allen's group has extensively studied the molecular basis of this antagonism and its effect on T cells in vitro and, more recently, in vivo.

The ability to recognize a range of similar peptides appears to be an essential feature of T-cell development. Thymic positive selection involves recognition of self-peptides that serve as surrogates of the foreign peptides that T cells encounter in the periphery during infection or vaccination. The following question arises: How does a T cell developing in the thymus not become activated in the periphery when seeing a related self peptide? To address this question, Dr Allen developed 2 strains of transgenic mice, with 1 strain expressing a TCR specific for a variant hemoglobin molecule for which an antagonist peptide was well known and the other expressing the antagonist peptide sequence.⁵ For technical reasons, the antagonist had to be expressed as a sequence inserted into another foreign antigen, which conveniently could serve as a control that the transgenic mice were properly expressing this chimeric antigen. By crossing the 2 transgenic strains, offspring mice could be produced that expressed the TCR on a majority of T cells and that were exposed from their earliest development in the thymus to the antagonist peptide.

Results showed that the presence of the antagonist affected the T-cell repertoire in an unexpected manner. Unlike a full agonist, which eliminates TCR-reactive T cells in the thymus, the antagonist was found to cause no gross change in the number of TCR-transgenic T cells. Examining the expression of the TCR on transgenic T cells with clonotypic antibodies, the only difference was a slight decrease in the number of T cells expressing the highest levels of the TCR transgene. In the periphery there was no difference in the levels of TCR expression. However, responses of these peripheral T cells to the full agonist peptide were inhibited. That is, compared with T cells of transgenic mice that did not develop in the presence of the antagonist peptide, responses were diminished 60% to 90%. However, further analyses revealed that these T cells were not anergized (rendered unresponsive). Purified T cells from the doubly-transgenic mice responded normally to the agonist peptide, indicating that the inhibition was the result of continued antagonism by APCs carried over into the cultures. Controls ruled out the possibility that sensitivity to antagonism resulted from poor expression of the antagonist peptide in the thymus—a transgenic TCR specific for an agonist that flanked the antagonist sequence in the chimeric molecule was strongly deleted in the thymus. These results support the interpretation that an antagonist peptide can positively select a specific TCR in the thymus and still antagonize the same T cell in the periphery. Further evi-

dence that TCR antagonism occurs in vivo came from adoptive transfer experiments in which TCR-transgenic T cells were injected into either normal or antagonist-expressing transgenic recipient mice. Immunization of both recipients with the full agonist peptide in adjuvant under optimal conditions resulted in a strong response in normal recipients but a greatly reduced stimulation in mice expressing the antagonist. Thus a TCR antagonist peptide can exert significant and prolonged effects on T cells in the body.

The precise mechanism by which antagonist peptides exert their effects on T cells is not entirely clear, but it is known to result from incomplete signaling by the TCR on interaction with the ligand-MHC complex. In current models of TCR recognition, this may be due to altered kinetics of the TCR-MHC interaction or may result in a different conformation of the MHC complex that differently triggers the TCR. In some cases the adhesion and signaling functions of the accessory molecule CD4 are needed to permit an altered peptide ligand to perform its effects. Together, these data suggest that the low avidity of the interaction underlies the effect.

Biochemical measurements of the affinity of the TCR for various ligands presented by MHC confirmed that antagonists are bound less strongly. A soluble version of the same hemoglobin-specific TCR used in the transgenic mouse experiments was produced. MHC molecules with covalently attached agonist or antagonist peptides were synthesized to permit study of TCR-MHC peptide complex interactions by the technique of surface plasmon resonance. Affinity measurements showed that the TCR interacted with the agonist-MHC complex with a dissociation constant (K_d) of 21 $\mu\text{mol/L}$, whereas the interaction with the antagonist complex was much weaker, perhaps as much as 10^{-4} lower. Although the actual avidity of the interaction at the cell surface is not known, clearly T cells can productively interact with ligands with much less binding ability than wild-type peptides.

These results show that T cells indeed live in a "noisy environment." Their ability to be positively selected by peptides of low TCR affinity in the thymus permits the development of a large T-cell repertoire, while avoiding autoreactivity by tolerance through antagonism. The ability of T cells to be antagonized by altered peptides appears to be an inherent aspect of their developmental program, and this approach may be eventually exploited to control unwanted T-cell responses in the periphery.

APOPTOSIS IN THE PATHOGENESIS AND TREATMENT OF IMMUNOLOGIC DISEASES

Dr Michael Lenardo (National Institute of Allergy and Infectious Diseases, Bethesda) focused on the role of apoptosis in the pathogenesis and treatment of immunologic disease. Apoptosis, also termed *programmed cell death*, results from triggering an internal program inside cells that results in fragmentation of cellular DNA, condensation of the nucleus, and blebbing of the cell into membrane-bound packets that are disposed of in the

body by phagocytosis. Important in understanding the control of immunologic diseases, T cells become susceptible to apoptosis immediately after antigenic stimulation through the TCR. This *proprioicidal cell death* is a major mechanism to prevent deleterious accumulation of lymphocytes in an immune response. There are 2 major triggers of T-cell apoptosis; signaling for apoptosis is mainly through the Fas molecule, but can also be promoted by TNF under certain circumstances.

Fas (APO-1, CD95) is a member of the TNF family, as is the corresponding Fas ligand (FasL or CD95L). Two strains of mice that exhibited similar autoimmune phenotypes were shown to have defects in this system of apoptosis, the *lpr* (defect in Fas) and the *gld* strains (defect in FasL). These mice highlighted the requirement for programmed cell death by Fas to avoid autoimmune disease, a finding that was later shown to occur in humans as well. The related human disease is termed *autoimmune lymphoproliferative syndrome* (ALPS) and is characterized by massive nonmalignant lymphadenopathy of early onset caused by the accumulation of α -positive, β -positive, CD4-negative, and CD8-negative peripheral T lymphocytes. More than 40 patients have been characterized and have been shown to have a defect in their lymphocyte apoptotic function.⁶

All patients with ALPS exhibit reduced TCR-triggered apoptosis. Most patients have a mutation in the Fas molecule (although a few do not, which requires further study). The Fas mutations usually cluster in the *death domain* of the molecule, the cytoplasmic region that interacts with another component of the apoptotic pathway, Fas-associated death domain protein, which was shown to be poorly recruited by Fas activation in the patient group. Mutations occurring near, but not within, the death domain usually resulted in a less severe defect, whereas mutants altered directly within the death domain exhibited a dramatic block in T-cell apoptosis. Patients with ALPS are usually heterozygotes, having 1 defective and 1 functional allele of Fas. Because Fas is a trimer and all the constituent monomers need to be normal to obtain function, only one eighth of the trimers that form in heterozygotes are fully functional, and seven eighths are defective. Thus the disease is a result of a dominant negative effect of the defective trimers on signaling apoptosis. The ALPS defect is most prominent early in life and then typically declines, probably as the thymus involutes with age and overpopulation by thymic export of T cells is reduced. Apart from explaining the basis of the disease, these studies allowed an accurate diagnosis of ALPS. Further research is needed as to the defects in those patients with ALPS who have no obvious mutations in Fas or FasL and on the other factors needed for disease penetrance because not all individuals with a mutated Fas molecule exhibit the disease.

In the second part of his talk, Dr Lenardo reported on his efforts to utilize receptor-triggered apoptosis in the antigen-specific elimination of pathogenic T cells. His research used the animal disease model for human multiple sclerosis (MS), experimental autoimmune

encephalomyelitis (EAE).⁷ Key features of EAE are the induction of CD4+ T cells specific for self-myelin proteins, demyelination in the central nervous system, and sensory and motor defects, which can have a relapsing and remitting course like that of MS. EAE can be induced either by immunizing with myelin components in Freund's complete adjuvant to stimulate autoreactive T cells or by adoptively transferring myelin-specific T cells into a syngeneic host.

Treatment of EAE by antigenic stimulation was studied. In a murine adoptive transfer model, injection of 30 million myelin basic protein (MBP)-specific T cells into a recipient produced a relapsing-remitting disease and typical central nervous system pathology. However, if MBP without adjuvant was injected into the recipient animals at several intervals after T-cell transfer, even after the first wave of disease onset, disease was abrogated. Mechanistic analyses with a transgenic T cell, which could be identified and characterized after transfer into the host, showed that the antigen injections led to the specific deletion of the MBP-reactive T cells by apoptosis. Thus an approach that may appear to be counterintuitive, injection of the target antigen, was able to dramatically reduce EAE, potentially a valuable strategy to treat MS.

Moving toward human clinical trials requires overcoming many hurdles, including the design of a version of human myelin for reproducible clinical studies and safety and efficacy information from preclinical studies on nonhuman primates. The antigen currently under study by Dr Lenardo and collaborators is a recombinant molecule, termed *MP4*, comprised of human MBP plus the T cell epitope-containing hydrophilic regions of proteolipid protein, another myelin component.

Disease studies were performed in the marmoset (*Calithrix jacchus*), a nonhuman primate that is susceptible to EAE induction and that can be monitored by the magnetic resonance imaging techniques used for patients with MS. In this model EAE was induced directly by injection of MP4 in complete Freund's adjuvant plus pertussis. The marmoset disease was very much like MS, with lesions showing loss of myelin sheaths, along with T cell and macrophage infiltrates, and paralysis. Several injections of the MP4 antigen alone resulted in lower disease scores and decreases in lesions observed by magnetic resonance imaging. Lower doses of MP4 reduced or delayed disease, whereas higher doses eliminated EAE entirely. No exacerbation of disease by the antigen treatment was ever seen.

These encouraging results suggest that the primate model will provide useful data on dosing and administration for human clinical trials involving patients with MS. To apply this therapeutic approach to other autoimmune diseases, the identity and nature of key T-cell antigens need to be defined and studied in appropriate preclinical models.

APPROACHES TO TRANSPLANTATION TOLERANCE

Dr David Sachs (Massachusetts General Hospital) reported on the induction of tolerance for transplantation.

Overcoming the barrier of immune rejection of transplants by induction of specific immunologic tolerance to the graft remains a major goal despite improvements in immunosuppressive strategies. Limiting tolerance to only the graft antigens would permit normal immune responses to pathogens, elimination of long-term immunosuppressive medication, and avoidance of many chronic problems seen in grafted organs, such as atherosclerosis in transplanted hearts maintained under immunosuppression.

Dr Sachs' laboratory has developed a tolerogenic approach based on a long-known strategy termed *mixed bone-marrow chimerism*. If, before an allograft, the recipient is totally immunosuppressed by high levels of irradiation followed by immune reconstitution with bone marrow from the prospective donor, the graft will be accepted. Two additional strategies are needed to prevent graft-versus-host complications and to achieve full ability for the recipient to defend against infection. First, all mature potentially alloreactive T cells must be removed from the bone marrow cells used for reconstitution. Second, reconstitution must include bone marrow from both the donor and the host to provide the full ability to recognize antigens presented by all APCs in the recipient. Mechanistically, bone marrow T-cell precursors from the donor become positively educated to accept as self the host MHC present on thymic epithelial cells, whereas T-cell precursors from the host become negatively selected by dendritic cells in the bone marrow derived from the future donor, resulting in tolerance to both the graft and the host. Experiments in mice proved that mixed bone marrow chimeras permanently accepted grafts from the same MHC haplotype as the non-self bone marrow donor, while maintaining the ability to reject third-party grafts.

However, this procedure is not acceptable clinically because of the toxic effects of lethal irradiation needed to ablate the immune system. Recent research in collaboration with Dr Megan Sykes (Massachusetts General Hospital)⁸ on approaches to avoid high levels of irradiation established that depleting T cells with mAbs coupled with much smaller amounts of radiation delivered to the whole body and to the thymus could accomplish the same depletion as lethal irradiation. Further studies indicate that whole body irradiation can be dispensed with if larger amounts of bone marrow are used to reconstitute the recipient. However, thymic irradiation is still needed because some mature T cells become antibody coated but still persist in the thymus. There is also no need in this protocol to specifically deplete the donor marrow of mature T cells because the anti-T cell mAbs used in ablation remain circulating and functional at the time of reconstitution. Animals treated by this procedure become mixed chimeras, fully tolerant of donor grafts while rejecting third-party grafts. The mechanism is believed to be the induction of central tolerance; staining of the thymus shows donor and host dendritic cells at the cortico-medullary junction where they could participate in T-cell repertoire selection. In support of this hypothesis, if development of chimerism is experimentally prevented by specific antibodies, subsequent grafts will be rejected.

The feasibility of this approach in nonhuman primates was explored. Before bone marrow reconstitution, cynomolgus monkeys were given 150 rads to the whole body and 700 rads to the thymus. Because of the lack of depleting mAbs for primate T cells, antithymocyte globulin was used, which does not eliminate T cells from lymph nodes and the thymus. Therefore a 28-day course of posttransplant treatment with cyclosporin A was needed to prevent acute rejection. Data showed that mixed chimerism occurred initially, and interestingly, animals remained tolerant to future donor grafts despite later losing chimerism. Dr Sachs has an institutional review board-approved protocol to test this approach in suitable human candidates. Currently, few recipients would be eligible for such a protocol, but this would not be the case for xenotransplantation, in which standard immunosuppression is not effective.

An exciting new approach using gene therapy to induce tolerance across MHC barriers was presented.⁹ This method involves transferring genes of the donor MHC to the recipient before transplantation by autologous bone marrow cells infected with a retroviral vector. This system has several potential advantages over mixed bone marrow chimeras. These include the following: only the genes for the foreign MHC are transferred rather than potentially alloreactive cells or antigenic molecules, the procedure may theoretically require milder conditioning (decreased irradiation may suffice), and a single or limited set of vectors may suffice for common donor haplotypes or inbred xenograft donors.

Initial studies used miniature swine inbred according to their MHC (SLA) haplotypes.¹⁰ Prior studies in that system indicated that matching for class II MHC is critical to obtaining graft acceptance. If class II is entirely matched, only a 12-day course of cyclosporin A was needed for permanent engraftment. It should be noted that pigs, like monkeys and humans, but unlike mice, express class II MHC molecules on vascularized epithelia, a major consideration in choosing this model for studying vascularized allografts. Briefly, a retroviral vector expressing the DR β -chain of strain SLA^d was used to infect bone marrow cells of strain SLA^c. Cells incorporating the vector were then selected by using a drug-resistance marker, and the infected cells were then injected into an irradiated SLA^c recipient. With initial immunosuppression, these swine were then able to accept SLA^d kidney grafts, whereas controls that received self-SLA^c were not tolerant. Research continues on discovering the best method to achieve high retroviral infection of the bone marrow cells; the use of growth factors in the cultures appears to be essential. The gene therapy strategy holds considerable potential for simplifying the induction of transplantation tolerance, and further research on the practical and theoretical aspects of the approach is being undertaken.

REGULATION AND COUNTER-REGULATION OF AUTOIMMUNITY BY CYTOKINES

Dr Nora Sarvetnick (Scripps Research Institute) focused on the role played by cytokines in the regulation

of the autoimmune process. Her studies primarily used the NOD mouse, in which a disease that is analogous to human insulin-dependent diabetes mellitus spontaneously develops. In both cases the disease results from cell-mediated destruction of the pancreatic islet cells, which produce insulin.

In general, T lymphocytes fall into 2 categories: T_{H1} cells, which produce IFN- γ and are important in inflammation and autoimmune tissue destruction, and T_{H2} cells, which produce IL-4 and IL-10 and are more important for allergic diseases and antibody production. IL-4 and IL-10 can also counter-regulate T_{H1} cells. Dr Sarvetnick's group has investigated the ability of cytokines to interrupt the autoimmune destruction of islets when the cytokines are introduced into the local vicinity of the islet.

Antibodies to IL-10 administered to NOD mice early in life block the development of diabetes. In contrast, when cells from a diabetic NOD mouse are transferred into an animal without disease, antibodies to IL-10 are unable to prevent the onset of disease. Thus IL-10 is required for the development of disease early in the process but is dispensable later. Similarly, if IL-10 is expressed in the islets by using a transgene, then there is a remarkable acceleration of the disease process. This acceleration is mediated entirely by CD8 T cells, as shown in studies with anti-CD8 antibodies. In contrast, IL-10 can inhibit the autoimmune process in the later stages, probably by inhibiting IFN- γ production from T_{H1} cells. These contrasting effects of IL-10, which depend on the stage of the disease, illustrate the complexity of the process.

One sees quite a different outcome when IL-4 is produced in the NOD islets by means of a transgene (ie, complete protection from disease and a complete block of the infiltrating insulinitis).¹¹ This effect is local because the mice still show inflammation in other sites, such as the salivary glands. The protection is due to a tolerogenic effect. This was shown in an experiment in which NOD islets that had been transferred to the kidney capsule of IL-4-transgenic NOD mice were not destroyed. This was true even though the IL-4-transgenic NOD mice still had T cells that could respond to islet antigens.

Mixing and transfer experiments established that the tolerogenic mechanism resided in the islets and not in the T cells. Thus if NOD T cells were mixed with T cells from an NOD IL-4-transgenic mouse and transferred together into an NOD, the cells from the transgenic mouse did not prevent diabetes. However, if diabetogenic cells from an NOD mouse were transferred into an NOD IL-4-transgenic mouse, the animal did not get diabetes. Moreover, other experiments showed that the T cells of the IL-4-transgenic mice had to have a diverse repertoire to see protection. Dr Sarvetnick believes this indicates that the mechanisms involved are complex and involve the resonance between the infiltrating T cells and the resident APCs.

The last cytokine reported on by Dr Sarvetnick was transforming growth factor (TGF)- β . When NOD mice express TGF- β in the islets by means of a transgene, dis-

ease development is reduced by approximately 50%.¹² Transfer experiments indicate that this was due to reduced pathogenicity of the T cells and that counter-regulation was not present in the islet tissue. Further studies revealed that TGF- β expression resulted in the following changes in the T cells: (1) a shift in T-cell preference for antigen presentation from B cells to macrophages; (2) a shift in their peptide recognition pattern; and (3) a polarization to a T_{H2} dominant response. In fact, the protection was dependent on this polarization and the secretion of IL-4. Thus TGF- β regulation is quite different than that produced by IL-4 itself.

DISSECTING THE B7-CD28/CTLA-4 COSTIMULATORY PATHWAY BY USING DEFICIENT MICE

Dr Arlene Sharpe (Brigham and Women's Hospital) spoke about the role of the B7-CD28/CTLA-4 pathway in the regulation of immune responses. She noted the critical importance of the pathway in that it has the unique capacity to prevent the induction of anergy *in vitro* and that interruption of this pathway by using CTLA4-Ig in animals can induce long-term graft survival and suppresses autoimmunity and allergic responses. It is a complex pathway that has 2 ligands (B7-1 and B7-2) and 2 receptors (CD28 and CTLA-4) and can result in both positive and negative signaling. Dr Sharpe and her colleagues have used the gene-targeting approach to develop mice deficient in B7-1, B7-2, or both B7 molecules or deficient in CTLA-4. She presented results on the effects of B7 molecule deficiency in humoral immune responses, in CTL responses, and in a mouse model of asthma, as well as the effects of CTLA-4 deficiency.

The antibody responses of mice to the T-cell dependent antigens trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH) or TNP-ovalbumin (OVA) were assessed in mice deficient in B7-1, B7-2, or both.¹³ When mice were immunized without adjuvant, B7-1-deficient mice showed delayed responses, but by 3 weeks after immunization they reached normal antibody levels. In contrast, B7-2-deficient mice immunized without adjuvant showed an absence of switching to an IgG-specific response and an absence of germinal centers. If the B7-2-deficient mouse was immunized with adjuvant, normal levels of antigen-specific antibody responses were achieved. Finally, mice deficient in both B7-1 and B7-2 failed to generate IgG responses and germinal centers even when immunized with adjuvant. Because the phenotype of the double B7 knockout mice resembled that of CD40 or CD40 ligand-deficient mice, it was reasonable to ask whether B7 was needed for the expression of CD40 ligand. T cells from mice deficient in both B7 molecules stimulated with anti-CD3 increased CD40 ligand expression initially, but then it declined. The addition of anti-CD28 could sustain CD40 ligand expression. Thus expression of B7 molecules is needed to sustain CD40 ligand expression. In summary of the humoral studies, B7 molecules are important for Ig class switching to IgG and for germinal center

formation. The roles of B7-1 and B7-2 overlap, with B7-2 having a greater role, and there is a critical interaction between the B7-CD28/CTLA-4 pathway and the CD40/CD40 ligand pathway. It may be possible to use B7 molecules themselves as adjuvants.

The role of B7 molecules in CTL responses was assessed by using primary antiallogeneic CTL responses where either the responder or stimulator cells were deficient in B7-1, B7-2, or both. If either responder or stimulator cells were deficient in one of the B7 molecules, then no effect was seen. If the responder cells were deficient in both B7 molecules, then there was a markedly diminished CTL response. However, if the stimulators were deficient in both molecules, then the response was only partially diminished. Addition of IL-2 could restore the responses. These results indicate that B7-1 and B7-2 have overlapping functions that are important for CTL generation, but neither alone is essential.

In collaboration with Patricia Finn (Brigham and Women's Hospital), Dr Sharpe has examined the effects of B7 molecule deficiency in a mouse model of asthma in which mice are immunized, boosted, and then exposed to antigen by means of an aerosol. The mice are then analyzed for airway hyperresponsiveness, IgG levels, and pulmonary inflammation. Absence of either B7-1 or B7-2 diminished all 3 responses, with the effect of B7-2 absence being greater. Preliminary studies with mice deficient in both molecules indicate a complete absence of allergic responses. These studies indicate that B7 molecules are important for allergic responses, with B7-2 having a greater role.

Mice deficient in CTLA-4 develop a fatal syndrome consisting of massive splenomegaly and lymphadenopathy, multiorgan lymphocytic infiltrates, and tissue destruction, and death by 3 to 4 weeks. Treatment of the mice from birth to 14 days with CTLA4-Ig prevents T-cell activation, but the syndrome returns after the CTLA4-Ig is stopped.¹⁴ This short-term effect contrasts with the long-term effects seen with CTLA4-Ig in transplant and autoimmune disease models and suggests a role for CTLA-4 in induction and maintenance of tolerance.

Crossing the CTLA-4-deficient mouse with the B7 double knockout mouse produced a mouse that had characteristics of the B7 double-deficient mouse, indicating that there does not appear to be additional B7 ligands in this model. In studies in which the CTLA-4 deficiency was introduced into a TCR-transgenic mouse, no impairment in thymus positive or negative selection was seen nor was there any alteration in activation-induced cell death. Thus it appears that the CTLA-4 inactivation signal primarily acts as a means of controlling tolerance in the periphery and that autoimmunity can result either from aberrant increases in B7 expression or decreases in CTLA-4 expression.

CD154-CD40 INTERACTIONS IN CELL-MEDIATED IMMUNITY

The role of CD154-CD40 interactions in cell-mediated immunity was presented by Dr Randolph Noelle (Dart-

mouth Medical School). He began by reviewing the characteristics of these molecules and the role of their interaction in humoral immunity. CD40 is a 50-kd integral membrane protein, which is a member of the TNF/nerve growth factor receptor family. It is expressed on B and pre-B cells, follicular dendritic cells, dendritic cells, endothelium, thymus epithelium, and macrophages. CD40 signals by means of TNF receptor-associated factor molecules. Its ligand is CD154, which is a 39-kd integral membrane protein and a member of the TNF ligand family. It is expressed primarily on CD4⁺ T cells and some CD8⁺ T cells. Studies in mice treated with mAb against CD154 and in mice deficient in either CD154 or CD40 provided insights into the role of the interaction of these molecules in humoral immunity. It was found in these systems that the interaction was essential for secondary humoral immune responses to thymus-dependent antigens and in the formation of germinal centers. Moreover, this interaction is also involved in the generation of memory B cells. In humans it was found that those individuals with hyper-IgM syndrome had mutations in the CD154 gene and consequently had little or no IgG, IgA, and IgE and no germinal centers. Thus these patients are profoundly immunodeficient and have serious and life-threatening infections.

Dr Noelle then reported on his studies of the role of CD40-CD154 interactions in cell-mediated immunity.¹⁵ The first system he reported on was the parent into F1 graft-versus-host disease (GVHD) model. Cells from parent b haplotype are injected into F1 (bx_d) mice and generate CTL against the host. The donor cells expand in a polyclonal inflammatory response, infiltrate the host tissues, and cause death in 20 to 25 days. If the mice receive a brief treatment with anti-CD154 antibody at the time of cell transfer, GVHD was not seen, and the mice did not die. This was also true if the donor cells came from a CD154-deficient mouse. Not only did the mice not die, but there was no evidence of subclinical GVHD by several criteria: (1) CTLs against the host were not detected *in vitro*; (2) there was no infiltration of inflammatory cells in the colon; (3) there was no increase in spleen cell number nor was there an expansion of donor cells; and (4) there was no increase in inflammatory cytokine levels in the colon.

Similar results were obtained in a second antiallogeneic CTL system in which allogeneic dendritic cells are injected into mice and CTLs are generated. Such CTLs did not appear if anti-CD154 was also injected or if the dendritic cells did not express CD40. Although the injected dendritic cells expressed high levels of B7-1, B7-2, and MHC molecules and produced inflammatory cytokines at the time of transfer, these characteristics were lost *in vivo* in the absence of a CD40-mediated signal to the dendritic cells. Similar results were also obtained in 3 other systems: (1) expansion of soluble antigen-specific CD4⁺ cells in response to antigen-pulsed dendritic cells; (2) the CTL response to GM-CSF-expressing melanoma cells; and (3) EAE. In each case anti-CD154 or cell populations deficient in either CD40 or CD154 prevented the response.

In contrast to the systems described above, other T-cell responses are not diminished by a lack of CD40-CD154 interaction. These include cell-mediated immune responses to certain viruses (eg, lymphocytic choriomeningitis virus and vesicular stomatitis virus) and helper T cell generation in response to antigens in the presence of a strong antigen. Thus it appears that chronic inflammation can generate mature APCs in the absence of CD40.

In summary, CD154 interaction with CD40 on APCs results in maturation of the APCs with upregulation of B7-1 and B7-2, production of both chemokines and cytokines, and the ability to function in a variety of cell-mediated immune responses. Dr Noelle is now evaluating what inflammatory signals can bypass the need for CD40 signaling in APC function. Humanized anti-CD154 holds considerable promise for the treatment and/or prevention of transplantation rejection and autoimmune disease in humans.

INHIBITION OF THE ALLERGIC RESPONSE BY IMMUNOSTIMULATORY DNA SEQUENCES

Approaches to inhibiting allergic responses were addressed by Dr Eyal Raz (University of California at San Diego). He reviewed the pathophysiology of asthma and other allergic reactions. There are 2 phases to allergic responses: the immediate hypersensitivity reaction and the late-phase reaction. Allergic asthma is primarily a late-phase reaction. T_{H2} cells orchestrate both types of allergic reactions. They produce IL-4, which stimulates B cells to make IgE and mediates the immediate hypersensitivity reaction by means of mast cells, and they produce IL-3, IL-5, and GM-CSF, which stimulate eosinophils that are the major mediators of the late-phase reaction. Eosinophils produce a variety of mediators, including major basic protein and leukotrienes, which induce the inflammation, tissue damage, bronchospasm, and airway obstruction that characterize asthma.

In the course of gene therapy experiments, Dr Raz and his colleagues demonstrated that immunostimulatory DNA sequences (ISS) within the plasmid (p) DNA played a pivotal role in the induction of the immune response to the gene product in gene-vaccinated animals.¹⁶ This ISS contains a CpG motif within a palindromic hexamer motif with the formula 5'-purine-purine-CG-pyrimidine-pyrimidine-3' (eg, 5'-GACGTC-3'). The ISS activate the production of IFN- α and IFN- β , IL-12, and IL-18 from APCs and IFN- γ from NK cells, all of which promote a T_{H1} response from naive T cells in response to antigen.

In the next series of experiments, Dr Raz and colleagues immunized mice with antigen alone and antigen plus ISS as an oligodeoxynucleotide (ODN). Compared with antigen alone, mice receiving ISS-ODN either by prepriming or coinjection produced high amounts of IgG2a antibody. When levels of IFN- γ were assessed, prepriming with ISS-ODN gave the highest levels.

They then did experiments to determine the effects of

ISS-ODN in a mouse model of allergic asthma.¹⁷ Mice are given 4 subcutaneous injections of OVA in alum at weekly intervals, followed 1 week later by OVA administered by inhalation and followed another week later with a second OVA inhalation. One day after the last inhalation, the mice were analyzed for eosinophil infiltration, levels of cytokines, and tissue histology. Such mice show high levels of eosinophils in bronchoalveolar lavage fluid, peripheral blood, bone marrow, and lung tissue. Administration of steroids for 7 days produces a moderate reduction of the eosinophils. ISS-ODN injected intraperitoneally before each inhalation completely abolished the eosinophilic infiltration. Control ODN had no effect. ISS-ODN administered only once before the first inhalation was almost as effective, whereas that administered once before the second inhalation was less effective. When the cytokines IL-5 (T_{H2}) and IFN- γ (T_{H1}) were analyzed, the ISS-ODN administered twice or once before the first inhalation showed marked reduction of IL-5 (88%) and induced large amounts of IFN- γ . Histology confirmed the results obtained by analysis of cytokines and eosinophils. ISS-ODN was also effective if administered intranasally or intratracheally, although somewhat less effective than when compared with systemic (intraperitoneal) administration.

In the course of these experiments, certain conditions of route and timing showed inhibition of the T_{H2} response (IL-5) without stimulation of the T_{H1} response (IFN- γ). Therefore splenocytes were incubated with ISS-ODN for 2 hours in vitro to activate the innate immune system and then plated in wells coated with anti-CD3 to stimulate T cells. Cytokine production was then assessed in the presence or absence of neutralizing antibodies to various mediators. ISS-ODN inhibited production of IL-3 (mediated by IFN- α and IFN- β), IL-5 (by IFN- γ), and GM-CSF (by IL-12 and IFN- γ).

On the basis of these and other data, Dr Raz proposed a 2-step scheme for the action of ISS-ODN. In step one no antigen is present, and therefore T cells are not stimulated. The ISS-ODN interacts with the innate immune system and stimulates APCs to produce IFN- α , IFN- β , IL-12, and IL-18 and stimulates NK cells to produce IFN- γ . These cytokines downregulate eosinophil induction. When antigen is introduced, these cytokines suppress T_{H2} induction and stimulate development of T_{H1} cells, which in turn produce more IFN- γ , which further inhibits eosinophil induction.

Perhaps the most interesting aspect of the results is the therapeutic implications. ISS-ODN is active when given in advance of the antigen. Therefore one does not need to know the allergen, and one does not need to run diagnostic tests to identify the pathogenic allergen. Moreover, the patient can be given the intervention irrespective of the timing of allergen exposure. Thus ISS-ODN has considerable promise for the treatment of asthma and other allergies.

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