

**STUDY OF NETWORK INTERACTIONS IN REGULATIONS OF
IMMUNE RESPONSE TO A MYCOBACTERIAL ANTIGEN: NETWORK
PERTURBATIONS OF CHRONIC GRAFT VS. HOST DISEASE CAN
INDUCE DELETION OF CD4+ TCELL CLONE SPECIFIC FOR M.
VACCAE BY CENTRAL AND PERIPHERAL MECHANISM**

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**SUBMITTED BY
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MUMBAI**

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And Science, we should insist, better than any other discipline, can hold up to its students and followers an ideal of patient devotion to the search for objective truth, with vision unclouded by personal or political motive.

--- Sir Henry Hallett Dalt



**Study of network interactions in regulations
of immune response to a mycobacterial
antigen: Network perturbations of
chronic Graft vs. Host Disease can induce
deletion of CD4+T cell clone specific for
M.vaccae
by central and peripheral mechanism**

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by
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INTRODUCTION



Capacity of the immune system to deal with infection is remarkable. The immune system can invoke many different effector mechanisms, with varying efficacy, against different types of infection. Innate immunity acts as a first line of defense against infectious agents and the adaptive immune system produces a highly specific response to each infectious agent which normally eradicates that agent. The adaptive immune response can take the form of specific cell-mediated immunity (CMI) or may involve the production of specific antibodies. Whether a CMI response or an antibody response is elicited will depend on a number of factors such as structure and amount of antigen, use of adjuvant, route of immunization and many others (1,2,3,4). Many immune reactions display both kinds of responses (5,6). The response pattern induced by a particular pathogen is usually predictable and often appropriate, but there are severe consequences if the incorrect pattern is induced which could lead to immune suppression, tolerance, anergy or death of immuno competent cells by apoptosis (7). The study of mechanisms leading to such consequences may help in combating the acquired disorders of the immune system or even in the design of a vaccine.

Specific immune responses result from the combined effects of specific antigen recognition and non-specific molecular interactions between different cells, which lead to the clonal expansion of antigen specific cells and their differentiation into mature immune effector cells and memory (8). The interaction between the antigen presenting cell (APC) and helper T (Th) cell is the first, and in many ways the most influential interaction in the evolution of an immune response (9).

Primary immune response

First exposed to a novel antigen, the immune system needs a considerable period of time before it begins making appreciable amount of effector response. Most antigen require some form of processing; for T cell recognition. The processing requirements are rigid i.e. the antigen is degraded into peptide fragments, which is performed by APCs. The processing involves binding of antigen to the surface of APC, internalization by phagocytosis or pinocytosis followed by active catabolic events which convert the antigen to a form which is recognizable in association with major histocompatible complex (MHC) molecules by T cells.

Secondary immune responses

Re-exposure to the same antigen produces a different response pattern, effector response and even antibodies appear within a period of hours. The frequency of T or B cells specific for previously encountered antigen is substantially increased by exposure (10,11,12,13,14).

Memory cells

Memory CD4+T cells appear to have less stringent requirements for activation than naive T cells (15,16,17,18). Markers which are lost or reduced upon activation of T cells are the high molecular weight isoforms of CD45R, a tyrosine phosphatase and the lymph node homing receptor MEL-14 (11,13,14,19,20). Several adhesion molecules are upregulated by activation including LFA-1, ICAM-1 as well as CD2, LFA-3 (12,13,21).

Subsets of Th Cell

A major advance responsible for much of the recent work on T cells and infection was the discovery, derived from the analysis of Th clones that CD4⁺ lymphocytes can be divided into two major phenotypic subsets, Th1 and Th2, based on their lymphokine secretion patterns (22,23,24). Th1 clones produce interleukin (IL) - 2, interferon (IFN) - γ and lymphotoxin or tumour necrosis factor (TNF) - β , the factors responsible for promoting delayed type hypersensitivity (DTH) reactions characteristic of CMI (22,23,24,25). Th2 clones produce cytokines such as IL-4, IL-5, IL-6, IL10 and IL-13 and were thus able not only to direct humoral responses, but also to promote allergic type responses with the activation of mast cells and eosinophils (22,23,24,26). Eventually it was also observed that a third subset of T-helper cells generates lymphokines of both Th1, and Th2 type. This subset was christened Th0 (27,28). It was then proposed that initial activation generates Th-expansion to Th-0 type which differentiates further to Th1 or Th2 cells with repeated antigenic stimulation (27,29).

Maintenance of memory cells

Recent studies of CD4⁺ subsets have radically challenged the prevailing paradigm that memory helper T cells are long-lived, continuously recirculating from blood to lymph nodes and capable of responding more quickly than naive cells (12,30,33). At the moment there is controversy about the extent to which external factors influence lifespan of memory cells (12,30,31,32). Whether an organism is cleared by the immune response or whether it can persist in the body, has profound consequences for the development and maintenance of memory (12,32,33).

Polar effector responses

Antigen persistence in the body can have polar effector responses. This is very well exemplified by leprosy. Leprosy, as a disease presents a spectrum of clinical responses paralleled by a spectrum of immunologic responses (6,34). At one pole of the spectrum- tuberculoid leprosy, there are few *Mycobacterium leprae* in the tissues and the patient develops high levels of CMI, vigorously producing IFN- γ (35,36). At the Lepromatous pole patients exhibit a selective unresponsiveness to antigens of *M.leprae* and the organisms ineluctably multiply in the skin, often to extraordinary numbers viz 10^{10} /g tissues. Between these poles the majority of patients exist in the border line categories- borderline lepromatous who are immunological unresponsive in general to antigen of *M.leprae* and borderline tuberculoid who immunologically respond to *M.leprae* antigens. Antibodies to *M.leprae* occur in all forms of disease but attain highest levels in lepromatous disease, suggesting that they have little to do with protection (36).

Immunosuppression

The immunological non-responder states observed in clinical situations are not uniform. The non-responder state can be complicated by suppression - a frequently encountered feature of chronic infection. In the past the reduction in immune responsiveness was associated with the appearance of CD8+ suppressor T cells (Ts) (37,38,39,40,41). These cells when adoptively transferred were able to specifically inhibit humoral and/or cellular immunity *in-vivo* (42). However, subsequent studies on Ts cells and their physiological role culminated in questioning their very existence (43,44,45). There was confusion of phenotypic markers on these cells and their MHC

restriction requirements led to controversies. Attempts at cloning the suppressors have also failed (38).

Earlier studies in our laboratory on the mechanism of non-responsiveness to *Mycobacterium vaccae* have shown that CD8+ T cells are expanded on intraperitoneal (i.p.) immunisation (4,41). These primed CD8 cells have been shown to suppress generation of DTH and lymphoproliferative response of the animals. Further our laboratory has reported that these cells are class II restricted and not class I as expected. Further the suppression mediated by these CD8 +T cells is achieved through competition with CD4+Th cells for the growth factor, IL-2 (46).

There are many other ways by which T cells can suppress immune responses in an antigen- specific manner. Polarised population of CD4+ T cell clones producing Th1 or Th2 cytokines have cross regulatory effects. Th1 cells producing IFN- γ inhibit responses by Th2 cells (47,48,49) and by production of IL-4 and IL-10 by Th2 cells down-regulate (suppress) inflammatory and Th1 responses (50,51).

Thus outcome of an immune response depends upon a complex network of interactions at the centre of which lies the clonally selected T or B cells responding to the antigen whose function an observer is interested in. The components of the network which play a major role, are:

- Antigenic epitopes, cross-reactive self antigens if any and the idiotope/s which form the mirror image of the antigenic epitopes.
- Quantitative and Qualitative aspects of antigen processing and presentation.
- Cognate interaction between the APC and the responding cell.
- Cytokines secreted by the APC.
- Presence of responding T/B or both clones belonging to a different functional subset and having the same epitope specificity.
- Nature of lymphokines secreted by these competing clones on activation.
- Capacity of these clones to compete for antigenic epitope and cytokines.

Activation and its relevance to outcome of immunization

Lymphocyte activation is a complex phenomenon. Current concepts on this issue have evolved from the original two signal model of activation proposed by Bretscher and Cohn (52). For example in T cells, activation is dependant upon signal one, delivered through clonally selected TCR, and other non clonally selected receptors such as CD4/CD8 and the second signal is delivered by CD28, the so called 'Co-stimulatory' receptor, and adhesion receptors such as LFA-1, CD2 etc (53). In case of B cells, the clonally selected surface immunoglobulin (Ig), and non clonally selected receptors such as CD40, CD23 are very important (54). In addition to the signals transduced through these, other signals delivered by lymphokines and cytokines also play a major role.

The different outcomes of immunisations such as sizes of effector and memory responses, subset of effector pathway selected,

anergy, apoptosis are dependent upon quantitative and qualitative aspects of these signals.

Large amount of knowledge enabling one to understand the cell-cell interactions, their control leading to a specific immune responsiveness has been accumulating during the last several years. Still numerous lacunae remain to be resolved.

Long term objectives of our investigative group consist of -

- Factors deciding size of effector and memory pool.
- Role of CD8⁺ T suppressors capable of competing for lymphokine IL-2 in deciding outcome of an immune response, in terms of effector and memory.
- Role of antigen presentation in deciding outcome of immune response in terms of effector and memory pool size and T cell subset differentiation.

In this particular work we are investigating two events.

Employing a model of chronic GVH we planned to study the role of antigen presenting cells in deciding the size of memory pool.

Chronic Graft versus (GVH) host disease model was used because in chronic GVH, parental T cells are activated by F1 alloantigens, to proliferate and produce lymphokines which in turn can activate macrophages and other APCs and B cells of the host without killing them (55,56). As a consequence there is chronic B cell proliferation and activation, alterations in the properties of cells capable of antigen presentation such as macrophages and B cells. This constitutes an environment of altered antigen presentation which can

be employed to study the effect of antigen presentation on the effector and memory pool size.

Further direction of this investigation was decided by published reports of loss of self MHC restricted responsiveness to foreign antigen in chronic GVH (57). The observation possibly implied that activation of CD4+ T cells of the host by antigen somehow induced loss of responsiveness.

Study the effect of endogenous superantigens on the ability of *M.vaccae* 65-kDa immunogen to expand CD8+ T cells capable of immune suppression by competing for IL-2.

In our earlier studies we have reported CD8+ T cells recognising a promiscuous epitope of *M.vaccae* 65-kDa antigen on MHC class II and inducing suppression by competing for IL-2(46). Since CD8+ T cells are class I restricted, the observation was curious. The APC presenting the epitope on MHC class II is not expected to provide proper activation because CD8+ T cells will not interact with MHC class II. Thus the question of what provides the requisite strength of interaction between the APC and the CD8+ T cells always remained an enigma. Since endogenous superantigens are ubiquitous in mice (58), we decided to investigate if these can provide the additional affinity of APC, T cell interaction by bridging the TCR V β of the CD8+ T cells and MHC class II of APC. If found true, then the finding would contribute an additional complexity to the ever widening network of interactions that influence the outcome of immunization.

REVIEW OF LITERATURE

Regulation of immune response to an antigen is of immense interest for a number of reasons. The regulatory mechanisms proposed have to account for a number of seemingly contradictory observations. Firstly, it is known that the animals are tolerant to self antigens and yet autoimmune diseases are encountered. Secondly introduction of antigen results into clonal expansion and yet after the exhaustion of antigen, the expanded size of the clonal population is again reduced. Thirdly introduction of antigen causes a variety of humoral or cellular responses. Each of these effector responses have further subsets such as antibody isotypes, T-helper effector subsets, cytotoxic T responses and others. Memory in clonally selected lymphoid cells constitutes another important phenomenon occurring after antigen administration. The present study was undertaken to explore the complexities of cellular interactions involved in T-helper cell activation. In order to place the objectives in proper perspective, the relevant literature is reviewed.

Outcome of Immunization

The introduction of antigen into an animal causes a complex series of events that result in a variety of responses. For descriptive purposes one can roughly divide an immune response into the humoral or antibody response and the cell-mediated responses. The basic requirement of the immune response must be that it is capable of recognition and responding with specificity to foreign agents, while maintaining an indifferent stance to self components. To achieve this a complex cascade of events, some developmental and others triggered by antigen, involving clonally selected as well as nonantigen specific multiple cellular subsets, occurs. There are several lacunae in our understanding of these events and the present

study attempts to address one such event on the regulation of clonal expansion after antigenic stimulation.

Activation - Overview

Immune activation normally involves a T or B cell in the resting G0 state entering cell cycle, proliferating and creating a clone of progressively more differentiated cells as activation proceeds ultimately generating effector cells. T and B lymphocytes recognize antigen in sharply different ways through their receptors. Antigen receptor engagement triggers the activation of a number of downstream signaling events including activation of Ras (59,60); hydrolysis of plasma membrane inositol phospholipids; increase in intracellular Ca^{+2} levels and activation of various protein kinases that subsequently phosphorylate a number of proteins involved in mediating the activation signals (61,62,63). Both, the Src -family tyrosine protein kinases Lck, Fyn, Lyn and Blk, and the Syk family tyrosine protein kinases Syk and Zap 70 coupled to the subunits of T cell antigen receptor (TCR) and B cell antigen receptor (BCR) play a role in lymphocyte activation (62,63). T cell activation results in cell-mediated immunity dependent on effector cell clones that exert their influence eg. lymphokine secretion or cytotoxic killing over a short range. B cell activation results in humoral immunity dependent on antibodies that travel in serum or secretions. However, it is important to regard these two major lymphocyte classes not as independent but as interdependent. The central event in generation of both humoral and cell-mediated immune response is the activation and clonal expansion of T helper (Th) cells (9). Activation induces the resting Th cell to enter the cell cycle and culminates in expression of the high-affinity receptor for IL-2 and secretion of IL-2. In response to IL-2 (and in some cases IL-4) (64), the activated Th cell

progresses through the cell cycle, proliferating and differentiating in to memory cells or effector cells.

The details of how the immune system divides its information for antigen receptors into multiple subunits are available even in textbooks. The recognition of antigen by B and T lymphocytes is now well understood although details continue to be provided, especially in the area of TCR interactions with peptide-MHC complexes.

Cell-Surface Molecules

The last decade has witnessed an explosion of insights into the molecular events which lead to the expression of a specific immune response. One aspect of lymphocyte receptor signaling that is becoming increasingly apparent is the remarkable similarity of T and B lymphocyte receptors (65). Both B and T cell receptors associate with invariant chains that are members of the immunoglobulin (Ig) gene superfamily of proteins CD3 γ, δ, ϵ for TCR (65,66) and Ig α (CD79a) and Ig β (CD79b) for BCR (67,68). These molecules have tyrosine-containing cytoplasmic motifs that allow association with cytoplasmic tyrosine kinases that are known to be involved in signaling (63,69). In addition, it is now clear that both TCR and BCR are aided in signaling by coreceptor proteins CD4 and CD8 in the case of TCR (70) and newly described complex of CD19-CR2-TAPA-1 in the case of BCR (71). CD4, CD8 and CD19 are all members of the Ig gene superfamily. In both cases these coreceptor complexes are associated with cytosolic signaling molecules, the Src family protein tyrosine kinase p^{56lck} in the case of T cell coreceptor (63,69,72) and phosphoinositol (PI)-3 kinase in the case of CD19 (73). Engagement of these coreceptors together with the antigen receptor potentiates activation by 100-fold in each case (74,75).

Finally CD45, the leukocyte common antigen found on all cells of hemopoietic origin, with its tyrosine-specific phosphatase activity plays a crucial role in the activation of both T and B cells (76,77,78). CD45 has the intriguing property of changing its ectodomain through alternative RNA splicing in T cells at different stages of maturation and differentiation (79,80,81). CD45 molecules with different ectodomains bind to different molecules on the T-cell surface. In naive T cells, CD45 has a high molecular weight form that does not bind either the TCR or the coreceptor (75,80). However, in memory T cells low molecular weight isoforms of CD45 bind to the TCR and the coreceptor (81). As these cells have altered signal transduction mechanisms and altered functions, it is tempting to speculate that CD45 regulates this process directly through its different intermolecular interactions (78,79,81).

T cell activation

The activation of T cells is required for virtually all immune responses. Activation can be assessed in many different ways, each illustrating a distinct facet in the complex process required for immune response.

The T cell receptor (TCR) is a multiprotein signaling machine and these protein subunits are compiled during T cell maturation and T-cell recognition to optimal signals leading to the later events. TCR comprising of hypervariable α and β chains that confer antigen and MHC specificity; a ζ family homo or heterodimers $\zeta:\zeta$, $\zeta:\eta$ or $\zeta:\gamma$ as well as the invariant chain receptor CD3 $\gamma\delta\epsilon$ alongwith CD4 and CD8 coreceptors and CD45 generate optimal signals for T cell antigen recognition (75).

Processing and Presentation

An essential step in the generation of T cell responses is the presentation of antigenic peptides by MHC molecules of APC to T lymphocytes, which can be divided into 3 steps. The first is antigen capture by the APC, this can occur either by binding of antigen to a specific receptor or by uptake in the fluid phase. This step is followed by antigen processing and formation of stable peptide-MHC complexes (82,83). In the presence of a sustained uptake of antigen and supply of MHC molecules, these complexes progressively accumulate on the cell surface (84). The third step is T-cell recognition, a process in which as few as 100 peptide MHC complexes (85,86) serially engage and trigger as many as 18,000 TCRs (87).

Remarkable progress has been made in elucidating the pathways that produce and supply peptides for MHC molecules. The general view has been that the MHC class I pathway is exclusively concerned with monitoring the endogenously synthesised proteins of a cell whereas the class II pathway presents exogenous antigens (88,89).

Several common themes in the molecular mechanisms that promote the assembly and function of both class I and class II molecules are becoming evident. For both classes of MHC molecules, molecular chaperons in the endoplasmic reticulum (ER) retain incompletely assembled complexes and prevent their degradation while at the same time maintaining them in an assembly component form. The invariant chain (Ii) functions in this manner for MHC class II molecules (90) whereas Calnexin, an ER resident protein is the chaperon for MHC class I molecules and probably also for incompletely assembled complexes of MHC class II molecules and Ii chains (91). Interactions of MHC molecules with chaperons also

ensure that the different classes of MHC molecules bind peptides in the right subcellular compartments.

The majority of peptides presented for MHC class I are generated by a phylogenetically old and conserved proteolytic particle, the proteasome which is responsible for degrading most proteins in the cytosol and nucleus (92). These peptides are then transported from the cytosol into the ER where they become available to newly synthesised class I molecules. This is accomplished by a peptide transporter associated with antigen (TAP) (93) a heterodimeric, MHC encoded ATP dependent transporter (94).

The MHC class II molecules predominantly present peptides derived from proteins occurring in, or directed to the endosomal compartment of the cell, Ii chain ensures that class II molecules bind peptides in the endocytic compartments by blocking the peptide binding groove and preventing the class II molecule from binding peptides in the ER (90,95). Moreover, the Ii chains contain sorting signals that direct the transport of the class II complex into endocytic vesicles where the Ii chain is released by proteolysis (96). Once free of the Ii chain, class II molecules can bind peptides a process which in many cases is further enhanced by the low pH in the vesicle (97).

TCR-MHC ligand interaction

T cells recognize antigen in the form of peptide fragments bound to the polymorphic cleft at the outer end of molecules encoded in the MHC complex. CD4 T cells recognize peptide fragments bound to MHC class II molecules while CD8 T cells recognize peptide fragments bound to MHC class I molecules. The structure of these complex peptide-MHC ligands has been established and the

investigators are studying the means by which these generate signals within a T cell.

Early studies of T cell activation utilising antibodies to the TCR revealed that TCR aggregation was required for activation (98). Relatively few TCR molecules probably less than 100 of the 50, 000 on a typical T cell need to be modulated by the ligand (99,100) for signaling to occur. Similarly relatively few of the 100,000 MHC molecules on the cell that is presenting the antigens to the T cell need to be bound to a specific peptide in order to trigger a T cell (85,86). T cells are extremely sensitive to low antigen concentration as only 30-100 peptide-MHC complexes on APCs are required for T cell triggering (85,86,99). There has been an estimate that the critical threshold number of peptide-MHC complexes to be in the range of 2-5 ligands per APC (99). On theoretical grounds, even one complex has been suggested to be enough for T cell triggering (100). This raises the question of how so few receptors can transduce an activation signal.

Interaction of TCRs with MHC ligands can have several effects on T cells, depending on the developmental maturity of the cell and the quality of the adhesion. The prevailing hypothesis is that immature T cells in the thymus are positively selected for maturation if they recognise cells expressing self-MHC complexes with a low binding affinity, such positively selected T cells are still susceptible to negative selection occurring following second recognition event in the thymus (101). If the molecular recognition event involves an affinity above a certain threshold, negative selection ensures heading to apoptosis-cell death (101).

The role of antigen density and avidity in directing T cell response has received considerable attention. The recent discovery of

TCR partial agonists and partial antagonists and the direct demonstration of variant patterns of T cell activation via TCR derived signals upon exposure to these ligands, have sparked a major change in thinking about the translation of antigen recognition into T cell activation (102,103,104). For mature T cells in the periphery strong interactions lead to activation whereas a weak one leads to anergy or non-responsiveness (106). Experimental evidence argues that in addition to intrinsic affinity, these outcomes are also directly related to the density on the APC a combined effect termed either efficacy or avidity (106).

Janeway (107), proposed that only the ligands that induce specific conformational changes in the TCR may be able to activate T cells. Different peptides as well as different anti TCR antibodies will have distinct effects on T cell activation because they produce different changes in the TCR conformation.

While these structural results raised new possibilities about the organisation of forming signal transduction complexes, there are other studies to explain the paradox of high sensitivity and low affinity of T cell antigen recognition. One such finding reports an unexpectedly large number of cycles of recognition and receptor activation mediated by a single peptide-MHC complex (87). It is suggested that, under physiological conditions T cells are triggered by sequential monovalent engagement of many TCRs by a small number of ligands. According to this model, a minimum time of ligation is required to assemble the multicomponent complex that transduces an activatory signal and is eventually down regulated. The re-use of peptide-MHC ligands in order to achieve efficient T cell activation implies that effective antigenic complexes must have an optimum affinity which allows efficient triggering and complex recycling.

Affinity and avidity are primarily determined by the interaction of the TCR with the antigenic peptide MHC complex. Other surface molecules, broadly, referred to as "accessory molecules" may influence the interaction of the TCR with its ligand by mediating adhesive interactions that control time, intensity and costimulation during the contact of the TCR with its antigen.

Accessory molecules

Activation of a T cell, although critically dependent upon the interaction of the TCR with its peptide-MHC ligand, actually involves a series of interactions with cell surface molecules on the APC. Some of those interactions may function mainly to strengthen the adhesion of the APC and the T cell, but others clearly provide important costimulatory signals. The interaction of B7 on the surface of the APC with CD28 on the T cell provides a signal which has received considerable attention (108,109).

At least four molecules expressed on the surface of APC (110,111), all members of the Ig-superfamily have been shown to costimulate T cell activation, and these are B7, intercellular adhesion molecule (ICAM-1 or CD54), Leukocyte function associated antigen 3 (LFA-3 or CD58) and vascular cell adhesion molecule (VCAM-1) (109,110,111,112,113,114). These costimulatory ligands function by interacting with their receptor expressed on the surface of T cells. Thus ICAM-1 interacts with CD11a/CD18 (LFA-1 / β 2 integrin) complex (112); LFA-3 interacts with CD2(LFA-2) molecule (111). VCAM-1 interacts predominantly with CD49d/CD29 (VLA-4/ β 1 integrin) (114) and B7 interacts with either CD28 or closely related protein cytolytic T lymphocyte associated antigen CTLA-4 (109,115). Adhesion is one of the clearly established functions of these

molecules, it is reported that even avidity of ICAM-1 and its ligand CD11a/CD18 and that of VCAM-1 and its ligand CD29/CD49d are increased upon activation of T cells (116). APC derived cytokines IL-1/IL-6 can also provide costimulatory signals that result in T-cell activation *in-vitro* (117). The heat stable antigen (HSA), which is a small phosphoinositol-glycolipid linked protein, can also costimulate the growth of naïve CD4 T cells (118).

Thus T cell activation in response to its antigen requires two signaling events (52,53,115,119). The first signal which confers specificity is mediated by the interaction of the TCR with antigenic peptide fragments presented by self class I or class II molecules encoded by the MHC (120). The second signal which induces clonal expansion is provided by the binding of a costimulatory B7 molecule to its receptor on T cells-CD28 (109,115). Signal two determines the outcome of the first signal, leading to either complete activation (115,119) partial activation (121) or to a long-lasting state of antigen-specific unresponsiveness termed anergy (122,123,124,125).

Two- signal Model

The two signal model of lymphocyte activation was first put forward by Bretscher and Cohn (52) for B cells and later by Lafferty (126) and Schwartz (115,119) for T cells. The two-signal model was originally devised to satisfy two conditions. First to account for the specific cell interactions that occur in the induction of the immune response and second, to provide an explanation of self-nonsel discrimination. The basis of the Bretscher-Cohn theory was that lymphocyte activation required two distinct epitope recognition: signal one entailed recognition of antigen by the specific lymphocyte in conjunction with a second recognition event delivered by an inductive antibody or cell binding to a second epitope displayed by

the same antigen. Thus in their model both signals were antigen specific (52). The Lafferty-Cunningham model proposed that the first signal was provided by the engagement of the antigen specific TCR and the second signal was a non-antigen specific costimulator signal provided by a metabolically active APC or stimulator cell (126).

However it is also becoming clear that the two signal model of lymphocyte activation is an oversimplification and that one may need to regard lymphocyte activation as a semiotic process in which the lymphocyte interprets signals delivered by its antigen receptor in terms of microenvironmental signs that include features of the antigen itself, costimulatory molecules and cytokines. Moreover, the interpretation of signals and signs may be influenced by the state of differentiation of the responding cells. Thus, the process of lymphocyte activation is in fact, highly complex and involves integration of many signals that together determine the outcome. Although cognate interaction of any of the several pairs of cell surface APC and T cell proteins lead to costimulation *in-vitro*, blockade of only B7-CD28 interaction produces anergy (127,128). Much attention has been focussed on the role of CD28-B7 since then. B7 surface receptors exist in at least three distinct isoforms, namely B7.1(CD80); B7.2(CD86) and B7.3 (129,130,131,132). B7.1 and B7.2 isoforms are expressed on a variety of APC including B cells, dendritic cells, Langerhans cells and monocytes (130,133). Expressions of mRNA for B7.1 and B7.2 on monocytes and dendritic cells can be rapidly induced following activation (131,132,133). Each provide signals at different points in the activation process (129,133,134). B7.2 appears to be the dominant costimulatory molecule during primary responses, whereas B7.1 which is upregulated later in immune responses may be critical in maintaining primary and secondary responses (133,135,136,137).

CD28 generates signals that are distinct from the TCR ζ /CD3 induced Ca^{+2} cyclophilin/calcineurin pathway (138,139). Signalling leads to the stabilization of mRNA for various lymphokines followed by an increase in transcription (138). This distinction suggested a potential complementation of signaling pathways induced by TCR ζ /CD3-CD4 and CD28. The absence of such complementation would lead to an incomplete signaling network. This would explain the fact that TCR ζ /CD3 engagement, without CD28 ligation, leads to suboptimal stimulation or a state of nonresponsiveness or anergy (105,122,128).

Although the overall response to stimulation by the B7 family is strong activation it has recently become apparent that triggering CTLA-4 has inherently different effects on T cell activation from that of CD28 (140,141,142). CTLA-4 and CD28 have similar lymphocyte restricted tissue distribution, and expressed only after lymphocyte activation (143,144), whereas CD28 is also expressed on resting lymphocytes (130,139). It has been shown that CTLA-4 binds B7.1 and B7.2 with much higher avidity than does CD28 (146), even though they have sequence homology and share common ligands (144,145,146). Recent studies have shown that CTLA-4 -high avidity receptor - is also a powerful negative regulator of T cell activation and autoreactivity (147,148). Still more and more information is coming from CD28 and CTLA-4 gene knockout mice and their role in development, activation and cell death (149,150).

Antigen presenting cell (APC)

Thus, APCs serve as an immunological window to the foreign world. In general, the various types of APCs can be divided into professional and non-professional cells. While the latter are found

among nonlymphoid cells, professional APCs such as dendritic cells (DC), macrophages and B cells form an integral part of the immune system.

There is growing evidence that different APCs use specialised mechanisms for antigen uptake (151). To take up antigen, B lymphocytes take advantage of immunoglobulin molecule expressed on the surface, whereas macrophages rely on phagocytosis (152). Macropinocytosis and the activity of the mannose receptor have been identified as efficient mechanisms of antigen capture in DC (153). The mechanism of uptake determines the intracellular compartment to which antigen is delivered and may determine the type of T cell epitopes generated.

DCs play a central role in antigen presentation. They capture antigen at peripheral sites and migrate to secondary lymphoid organs where they trigger naive T cells (154). In addition to the efficient antigen capture, DCs fulfil other requirements for antigen presentation and T cell stimulation : they synthesize high levels of class II molecules, possess a very prominent class II compartment (155) and express abundant levels of adhesion and costimulatory molecules. These features allow DCs to perform as the most efficient APCs. In contrast to B cells and macrophages, dendritic cells constitutively display costimulatory activity for CD4⁺T cells (154,156). Accordingly, dendritic cells are 10 to 50 fold more potent than monocytes or B cells in inducing T cell responses to a panel of superantigens and require lower doses of antigenic ligands than do other APCs to stimulate T cells (157). Dendritic cells are more efficient inducers of proliferation and cytokine secretion (IL-1, IL-2 and IFN- γ) in allogenic mixed lymphocyte reactions as compared to macrophages and B cells (158). These functional differences may reflect the specialization of each type of APC. Macrophages and B

lymphocytes have to receive activation signals [bacterial walls, Lipopolysaccharide (LPS), mannan glycans etc.] to become costimulatory (159) and to present productively antigen from intracellular or extracellular pathogens respectively.

Different antigen-presenting cells elicit responses in different T cell populations for primary activation, secondary stimulation and cytotoxic effector functions (160). Maturing bone marrow derived dendritic cells may acquire and process antigens, transport them to lymph nodes and activate naive T cells there. By contrast, follicular dendritic cells, acquiring antigen antibody complexes, maintain memory via B-cell activation (161,162).

The B cell is an efficient APC because of its ability to concentrate antigen via its antigen-specific receptor and its expression of various accessory ligands which stimulate T cells (152,162). The specificity of the interaction between T and B lymphocytes is determined by peptide fragments of antigen bound to B cell MHC class II molecules and the T cell antigen specific TCR (54,163,164). Antigen specific interaction mediated by MHC class II and TCR can lead to the activation and proliferation of both B and T cells, lymphokine production by the T cell and differentiation of the B cell into an antibody secreting plasma cell. Intercellular communication events which drive these responses are mediated not only by MHC class II and TCR but also by other cell-membrane associated ligand-receptor interactions, as well as soluble interleukins (164,165).

B cell stimulation

The B cell receptor complex comprises the clonotypic antigen-binding component, surface Ig and its accessory transducing molecules Ig α and Ig β . Ligation of the BCR induces protein tyrosine

kinase activity and hydrolysis of phosphoinositol biphosphate. Specific recognition of foreign antigen by cell-surface Ig induces B cells to proliferate and differentiate either into plasma cells, which produce soluble immunoglobulin to fight infection or into memory B cells which can respond to subsequent encounters with the same antigen. This process however, requires help from activated T cells.

Signal transduction from the antigen receptors on B and T cells generally involves similar or identical molecular mechanisms. However, these receptors so far appear to be different in one aspect while the TCR interacts on the T cell surface with other molecules such as CD4, CD8, CD2 and CD5, no such coreceptors have yet been found for the BCR. Recent data reveal that several proteins on the B cell surface are either modulated or phosphorylated after BCR cross-linking. These proteins which are candidates for coreceptors are CD19 and CD22. Antigen receptor signaling can be modulated by CD22(168) and CD19/21 (167), which act to lower the threshold of antigen stimulation required for B cell activation (74,166). The CD19 molecule forms a complex with three other proteins namely Leu-13, TAPA-1 and CR-2 complement receptor in the plasma membrane (71). CD20 also regulates BCR signals; since cell-cycle progression requires precise levels of $[Ca^{2+}]$, the finding that CD20 is itself a Ca^{2+} channel provides a rationale for CD20-mediated modulation for both positive and negative signals of B-cell activation (168). $Ig\alpha/\beta$, CD19 and CD22 are all substrates for the protein tyrosine phosphatase (PTP) CD45 (76). CD45 expression is also required for full BCR signalling (79,169).

Nearly 25 years ago, researchers noted that efficient humoral responses to the majority of antigens require the concerted action of T and B cells. Once a Th cell recognizes a processed antigen displayed by a class II MHC molecules on the membrane of a B cell the two cells

interact, forming a T-B conjugate. More recently, it has become apparent that T-cell help for B cells comprises both soluble mediators-cytokines and membrane expressed stimulatory molecules. After antigen-specific activation, increased expression of many B and T cell surface adhesion molecules such as CD28/CTLA-4:B7.1/B7.2; LFA-1(CD11a/CD18):ICAM-1(CD54); LFA-2(CD2):LFA-3(CD58); VCAM(CD106):VLA-4(CD49d/29) and CD40:CD40L(gp39) serves to stabilize T-B cell conjugation (54,162,165).

It has been suggested that clustering of TCR, CD4 and LFA-1 molecules at the junction of an antigen specific T-B conjugate may increase the avidity of the cellular interaction. This increased avidity would prolong the association between the Th cell and B Cell, providing time for additional membrane signalling events and for the directed secretion of various cytokines (54,162).

Soluble T cell derived cytokines enhance B cell activation and proliferation in this manner and are essential costimuli for subsequent B cell differentiation and Ig production (170). The role of such cytokines IL-2, IL-4, IL-5, IL-6, IL-10, IL-13 and IFN- γ in B cell activation and differentiation has been studied extensively (9,164). IL-4 can reduce the threshold of sIg-mediated PKC activation (171). Although cytokines alone can induce early activation events in B cells, no cytokines or combination of cytokines induces clonal expansion of small resting B cells. There appear to be necessary short-range or contact dependent signals delivered to the B cell when it presents antigen to T cell (164).

The recent identification of CD40 ligand (CD40-L-gp39) expressed by T cells has helped to elucidate in molecular terms the critical cell-surface molecule on activated helper T cells that provides

help to B cells (172). CD40 plays a central role in proliferation of T cell dependent B cells. Ligation of CD40 can, depending on circumstances drive resting cells into the cell cycle, reduce the threshold for sIg signaling, rescue germinal centre (GC) cells from death (apoptosis) and promote Ig isotype switching (173,174). Some antigens however, can also stimulate B-cell activation and isotype switching in the absence of CD40L or T cells. Cytokines derived from non T cells, such as natural killer cells, macrophages and mast cells, have been claimed to be responsible for isotype switching in T cell independant response (165).

During an immune rseponse, B cells fulfill an important role, not only as antibody producing cells but also as APC (152,162). Which may even adversely affect the immune response. Indeed a number of investigators have suggested that T cell anergy or deletion *in-vivo* results when antigen is encountered on B cells that lack accessory molecules (175), since the levels of B7.1 and B7.2 on resting B cells are inadequate for reciprocal T cell activation, the signals that regulate the expression of B7 indirectly regulate T cell activation by controlling the APC function of B cells. CD40L can induce the upregulation of B7.1 and B7.2 on resting B cells (176,177). Although originally identified as a costimulatory molecule for antibody responses, the role of CD40L in the regulation of APC function and the expression of other co-stimulatory molecules focuses attention on this molecule as a decisive factor in tolerance versus immunity (174).



Effector Responses

During the 1970's, it was suggested that the different types of responses the cell-mediated and the humoral, may be attributable to heterogeneity of CD4⁺T cells, and this concept was strengthened during the 1980's by the observation that mouse CD4⁺ T cell clones can be subdivided according to two predominant cytokine secretion profiles (22). Mouse Th1 cells produce IL-2, IFN- γ and lymphotoxin whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (23,24). The function of Th1 and Th2 cells correlate well with their distinctive cytokines. Th1 cells are involved in cell-mediated inflammatory cytokines: several Th1, cytokines activate cytotoxic and inflammatory functions (23). Th1 clones induce delayed type hypersensitivity(DTH) reactions; and IFN- γ is commonly expressed at sites of DTH reactions (25). Conversely Th2 cells help in antibody production and particularly IgE which play a prominent role in anti-helminthic and allergic responses (23,26,178).

Development of the appropriate Th subset during infection is important because certain pathogens are most effectively controlled by either a predominately cellular (Th1) or a humoral (Th2) type immune responses (179,180,181,182). In some chronic diseases, dominance of the improper T helper cell response can exacerbate the disease and lead to an inability to eradicate the invading organism. For example, tuberculoid leprosy is accompanied by a strong delayed type hypersensitivity or Th1 type response that ultimately kills and clears the bacilli, although inflicting immunopathology (183). In lepromatous leprosy, on the other hand weak cell-mediated immunity, combined with the production of Th2 type cytokines results in the organisms multiplying, so that a much more severe disease persists (34,35).

A number of important *in-vivo* and *in-vitro* observations have helped elucidate the differentiation process that directs a naive precursor Th(Thp) cell which makes only IL-2, down a particular maturation pathway to become either a Th1 or a Th2 cell (29,185). Initially CD4+ T cells are thought to correspond to Th0 clones that simultaneously make lower levels of Th1, or Th2 type cytokines (27,28,29) but as the response begins to polarise towards either Th1 or Th2, polarization is reinforced because each subset has an autocrine response favouring its own pattern of cytokine commitment that further act to suppress the development of the opposite effect (48,49,50,184,185). Even a single cell clone has been reported to develop into either Th1 or Th2 cytokine secretion pattern (186).

Many studies have indicated that different microbes or protein antigens administered under different conditions routes (1,4) or doses (2,3) appear to favour the selective induction of distinct Th subset and thus the development of humoral or cell-mediated immune responses (24). Early *in-vivo* studies showed that both low and high amounts of antigen primed for DTH, whereas moderate levels stimulated antibody production (1). Recent experiment *in-vitro* have also implicated antigen dose as an important factor in the Th1, Th2 decision, although the interpretation of the results is complex. Increasing antigen dose may switch the immune response from Th1 to Th2 or vice versa and when a wide range of antigen concentration is used, two switching events can occur Th2 - Th1 - Th2 (135,187). However, results *in-vivo* (1) suggested a DTH - antibody - DTH progression. The consequences of 'low' or 'high' antigen doses may depend on the soluble or particulate nature of the antigen. Soluble form of antigen favours Th1, kind of response whereas particulate type favours Th2 kind (188). TCR-MHC interactions also influence

the kind of response stronger TCR-MHC interactions favours Th1 but weaker interactions favour Th2 response (189).

Factors which influence the differentiation of Th0 cells in to cells with Th1 or Th2 phenotype include not only the nature and amount of antigen, but the involvement of a particular APC (135) as well as the production of particular cytokines by either accessory cells/APC or T cells in the microenvironment which play an important role in the development of Th cells producing discrete arrays of cytokines. Cytokines which have been found to alter Th cell phenotype are IL-4 and IL-13 and to a lesser extent IL-10 which induce the development of Th2 cells (29,50,64,191,192) whereas IL-12, IFN- γ enhance Th1, development (29,194). The cytokines secreted by helper T cells affect not only overall antibody production, but also the type of antibody made by B cells IL-4 stimulates B cells to make IgG1 and IgE whereas IFN- γ tends to induce B cells to make immunoglobulin of the IgG2a type in mice (195,196).

Other cytokine patterns and sources

Although there are well documented Th1, and Th2 responses these are not the only cytokine patterns possible. T cells expressing cytokines of both patterns i.e. the Th0 patterns (27,28); cells producing high amount of TGF- β are termed as Th3 (197) and some additional phenotypes have been described among long-term clones.

Although T helper cells are the major sources of Th1 and Th2 kind of cytokines, many other cells within and outside the immune system also produce these cytokines NK cells produce IFN- γ and TNF and contribute to Th1 like responses (198). IL-4 and possible Th2 cytokines are synthesised by mast cells, B cells, Basophills (199). IL-10 is produced by macrophages, keratinocytes (24,190).

CD8⁺T cells often secrete a Th1 like cytokine pattern. There is now increasing evidence for the existence of Th2 like CD8⁺ T cells in humans and mice (34,200,201). Thus several cell types may contribute to an overall Th1, or Th2 cytokine pattern and it has been suggested that these responses should instead be described as type 1 or type 2 responses (134,135,202).

IL-12, a heterodimeric cytokine produced primarily by phagocytic cells has received much attention during past few years as an immunoregulatory cytokine (203,204). In addition to favouring the differentiation of Th₁ cells from naive precursor, IL-12 acts as a second-signal growth factor for Th₁ but not Th₂ to Th₀ clones (205,206). IL-12 plays a significant role in promoting endogenous protective responses during infection and/or contribute to pathology resulting from unregulated cytokine expression. It contributes to early defense during certain bacterial infections and also by facilitating development of Th₁ provides late protection (204). In contrast, down regulation of IL-12 during acute infections can be a key event in the establishment of chronic infection and protection against harmful excessive cellular immune response (204).

It was rapidly realised that many cytokines have multiple cellular sources, multiple targets, a broad overlapping range of activities and function in a network as a complex signalling language. This network is further complicated by reciprocal regulatory processes.

Recent progress centers on the network of cytokines, accessory molecules and cell types that shape the differentiation of distinct T helper cell subsets. The role of costimulatory molecules in selectively driving Th - cell maturation is an important new area. Mice deficient

in CD28 appear to have intact CMI but impaired humoral responses (207). Antibodies against B7.1 or B7.2 selectively inhibit the development of Th1 and Th2 responses respectively, both *in-vitro* and *in-vivo* (133,137,208,209). The relative expression of B7 isoforms on APC may also be critical in determining the nature and extent of the immune response (133,134,136,142,209). Immunoregulatory cytokines may influence the development of the Th1, versus Th2 immune responses by altering the levels of B7 isoform expression on APC. In particular, IL-10 IL-12 have been suggested to elicit their biological effects via B7 and CD28 molecules respectively (210 212). Cytokines which induce the development of predominantly the Th2 cells (eg IL-4, IL-10) inhibited the expression of B7.2 and moderately enhanced the expression of B7.1. IFN- γ which induces the development of Th1 cells, enhanced the expression of both B7.2 and B7.1 isoforms (129).

Greater understanding of the role of B7 isoforms in immune regulation may lead to development of novel therapies for a range of conditions such as AIDS, transplantation and autoimmunity.

Secondary Responses

Activation extensive proliferation and differentiation in to primary effectors is the first outcome of an effective immune response to an antigen. Primary immune responses of T and B cells are generally followed by a state of long-lived memory where secondary contact with antigen leads to a more intense response than the primary response. Memory is a phylogenetically conserved and fundamental feature of vertebrate and invertebrate immune system. Immunological memory is long lasting and is carried by both T and B lymphocytes.

Immunological memory is explained by the fact that after primary exposure to antigen, elevated number of antigen specific lymphocytes ie. memory cells remain in the host (11,12,14,212,213). Reexposure to the same antigen or pathogen then induces a faster response which is early enough to prevent widespread pathogen replication, so that the disease can be prevented. However, definition of a discrete memory population is difficult. The pathways involved in memory T cell generation are also unclear. There is emerging consensus that most specific lymphocytes involved in a response eventually die of apoptosis, but how a cohort is induced to develop into memory cells is unclear (214,215).

The role of antigen in maintaining memory is still controversial, although quite a number of the reports suggest that the persistence of antigen specific memory appears to be independent of continuing exposure to antigen (30,31,32,216).

A plethora of surface molecules has been proposed as markers of memory, over recent years. Most appear soon after activation and may not be stably expressed over long periods of time (217,218). Swain and colleagues who are striving to differentiate between naive, recently activated, memory and effector cells, make the very pertinent point that the definition can only be made by considering phenotype, activation state and analysis of function including cytokine production and helper activity (219). Relevant points are reviewed to explain few facts about the memory population.

CD4+ T cell Memory

A large portion of the phenomenon of memory is due to the fact that an increased frequency of antigen responding cells exist following initial exposure to antigen and that the memory lymphocytes have a set of properties which enable them to respond to reenconter with antigen in a much more vigorous and effective way. It is crucial to understand the factors which promote the differentiation of lymphocytes and which in particular promote their transition to memory instead of death by apoptosis.

The phenotypic characteristics of lymphocytes are useful in aiding in the discrimination between subsets and between stages of development. Indeed the naive, memory and effector populations can be distinguished on the basis of a plethora of different cell surface markers and some of these correlate clearly with their functional activities.

The small resting naive CD4 cell express low levels of many potential adhesion and activation markers (220). Although they express high levels of TCR/CD3 complex, they express only low to moderate levels of the integrins LFA-1 CD18a/CD54, Ig-superfamily receptors for intengrins such as ICAM-1 and they do not express costimulatory molecules such as CD40L (221). They express few receptor for cytokines which drive T lymphocyte growth IL-2, IL-4 and they express low levels of hyaluronate receptor CD44 (219,222). In addition they express high levels of L-selection, the lymph node High endothelial venule (HEV)-binding molecule involved in their continuous recirculation (223,224) and high levels of the high molecular weight forms of CD45 phosphatase (225,226). Resting naive CD4 cells initially produce only IL-2 in response to stimulation

(227,228). Following activation naive phenotype shifts with numerous changes. IL-2R is upregulated and other cytokine receptors are also upregulated (229). CD44 is upregulated and there is increased expression of several integrins including LFA-1 (230). CD40L is upregulated transiently (231). The cells become larger progress through cell cycle. L-selection expression declines, while expression of other adhesion molecules involved in homing and migration to tissue increases (230,232). Bradley and Watson (233), have studied the regulation of lymphocyte homing, recirculation and localization or migration during immune response. They show that different combinations of adhesion molecules and/or their ligands contribute to differences in recirculation of cells at different stages of development and to the selective recruitment of subsets of cells to tissue sites of inflammation.

Effector cells are large blastoid cells, with high expression of CD44, LFA-1, IL-2R and other activation associated markers (219,234). Expression of CD45RB varies depending on how the effectors were generated (235,236). A definitive marker of effector cells different from activation markers in general is lacking. Fas and FasL molecules which can be involved in autonomous death, while low on naive cells, are highly expressed by effectors (237) and other Tumor necrosis factor receptor (TNFR) superfamily members such as CD27 and CD30, as well as CD40L implicated in B cell help are also expressed on effectors (238). Recent studies have shown that CD40L which transiently expressed by activated CD4 cell (231) and which regulates B-cell responses, induces endothelial cells to express CD40, E-selectin and ICAM-1 (239).

After the effector response has subsided, resting memory T lymphocytes are found. The transition from activated cell to resting

CD4 memory is accompanied by maintenance of CD44 (222) complete loss of L-selectin (19) and usually by downregulation of the molecular weight isoforms of CD45R (20,226). Thus allowing a clear distinction from the naive lymphocytes which express the reciprocal pattern. However Westerman and pabst (240) argue that this is indirect evidence. The only antibodies available to determine the memory phenotype are for human T cells (223). In humans two distinct types exist naive phenotype express CD45RA^{hi} and memory CD45RO^{hi} (20). In mice, rats and sheep the CD45R isoform of naive T cells is identified, however memory T cells are characterised by default, as being non-naive T cells. This is a problematic approach, since it is known from human T cells that they acquire memory phenotype and function without down regulating the naive phenotype (240).

There is clear evidence in rats, and indications in humans, that the phenotype taken to define memory T cells can revert back to naive phenotype (217,218,242). This questions the relevance of such markers because by definition, a memory T cell can never be a naive T cell again. There are no known cell surface phenotypic differences which distinguish primary and secondary effectors. Upon initial restimulation, resting CD4 memory cells sampled from normal animals on the basis of phenotype and resistance to adult thymectomy make IL-2 as a predominant cytokine, in titers roughly equivalent to naive CD4 and produce low titers of IL-4, IFN γ and other cytokines. Swain *et al* (244) have shown that it is possible to generate Th1 and Th2 -polarised memory CD4 T cells which make patterns more reminiscent of the polarised effectors.

Generation of memory effectors from resting memory cells is quicker *in-vivo* than that of primary effectors from naive cells(2-3

days as exposed to 4-5 days) (229,245). As memory cells differentiate into effectors they again become capable of synthesis of very high titers of cytokines (229). Memory or secondary effector populations can be extraordinary efficient helpers. They also have altered expression of adhesion receptors and circulatory patterns (239).

Charles Mackay, first introduced the concept that 'naive' T cells selectively migrate into lymphoid organs and memory T cells migrate into nonlymphoid organs (246,247). Moreover, the concept has become generalised and is reported as established fact, even though it is based mainly on indirect evidence obtained in one species. Neither preferential accumulation of T-cell subset nor preferential expression of specific adhesion molecules-ligand pairs are sufficient to infer specific migration routes of naive and memory cells (233,240). Lymphocyte adhesion does not automatically lead to lymphocyte migration into a tissue: *in vivo* videomicroscopy has shown that many lymphocytes adhere to HEVs for seconds, but only approximately a third adhere and migrate into the endothelium (248). Studies have shown that in sheep and rats *in vivo*, naive T cells migrate both into lymphoid organs and into the skin (249,250) and memory T cells migrate into nonlymphoid organs such as the skin and also into HEVs and efferent lymph (251).

B cell Memory

The development of B cell memory is a T cell dependent process (14,30). Memory B cells are generated from centrocytes in the apical zone of germinal centers. These are foci of intense antigen driven proliferation that appear towards the end of the first week of the immune response (14). GCs are initiated by a small number of precursor cells (252) that have almost certainly been activated

previously in the T zones of secondary lymphoid tissues (253). The T zone proliferation is primarily involved in generating the primary antibody response, but a few of the cells activated there migrate to follicles and start to proliferate.

CD40 signals act at two distinct points in the differentiation pathway to allow the entry of cells in to the memory pool (254). The first, delivered within a few days of antigen encounter, drives proliferation and causes a rewiring of the molecule such that it can now transduce survival signals. The second CD40-mediated event that takes place in the GC, at the point of exit into the memory pool. Unless at this stage a GC B cell can elicit and interact with CD40L on T cell interact with CD40L on T cells around the GC it will die in situ (255,256,274).

Somatic mutation and selection on the basis of antigen-binding affinity progresses in the dynamic microenvironment of the germinal center in the pathway to memory B-cell development (255,257). Memory B cells differ from their naive counterparts in a multiplicity of characteristics including [a] their increased triggerability even with cross reactive determinants (15), [b] their clonal expansion potential (10,258), [C] their homing and recirculatory patterns (259) and [d] their expression of surface Ig receptors of isotypes other than IgM or IgD (260,261,262) that generally display increased affinity for antigen (263,264,265) and amino acid sequences that are altered by the accumulation of somatic mutations (264,265,266,267).

While naive B cells only express IgM and IgD, memory B cells express additional isotypes, including IgG, Ig A and Ig E (11,13,14,33). In addition, the level of IgD often appears to be reduced on memory B cells (260). Another membrane marker that appears to

distinguish naive and memory B cells is an antigen designated JIID. Naive B cells express high levels of JIID, where as memory B cells express little or no JIID (268). In addition. memory B cells express higher levels of various adhesion molecules eg ICAM-I than do naive B cells.

Little is known about the process of memory-cell formation and 3 models have been proposed (270,271,272). The most widespread model is that memory B cell and plasma cells develop from unequal division of a common precursor cell (270). A second model is that cytokines produced from T cells or various accessory cells may influence the differentiation of a common precursor cell into a memory cell or a primary antibody forming cell (271). A third model, which is gaining support, is that memory cells and plasma cells arise from different lineages, each of which clonally expands following primary antigen exposure (272).

Life-span

Although specific immunity following an infection can last for years or even a lifetime, some pathogens/vaccines induce only short-term protection. The physical properties of antigen, in-vivo persistence, route of exposure, dose and frequency of reexposure can influence the length of immunity (273,275,276). Swain and her co-workers showed that in mice, memory T cell function is found in a long lived population of cells that once generated, is unaffected by adult thymectomy which depletes naive cells (219). However other studies suggested that individual memory T cells may be relatively short-lived and turn over more rapidly than naive T cells (218,276).

Two different mechanisms have been postulated to account for immunological memory. The conventional model holds that specialized long-lived memory cells harbour memory (32,213,270,277). However, the formal demonstration of long-lived antigen specific memory cells has proved difficult. Most of the older studies demonstrate the persistence of memory in the absence of cell-division. Schittek and Rajewsky (278), have demonstrated the persistence of memory in absence of cell division in mice using techniques to label proliferating cells. In another study Mullbacher (216) has demonstrated that CD8+ memory T cells reactive to influenza virus can persist for 25 years in the absence of antigen.

The second model assumes that memory is critically dependent on continuous stimulation by the antigen. In adoptive transfer experiments memory B cells have been shown to decay rapidly in the absence of antigen, while they survived indefinitely when transferred with the antigen (275). Similar dependence was reported for T cell memory as well (276). Follicular dendritic cells (FDC) are proposed to play a critical role in immune memory responses. FDC within the primary follicles and Germinal Centers(GC) serve as antigen depots and constantly restimulate B cells (280,281). However, FDC can not process antigen and therefore can not participate in maintenance of T cell memory. But it has been shown that B cells can become effective as APC when interacting with FDC in an antigen-specific manner (161).

Studies showing decay of T cell memory in the absence of antigen suggest that memory cell survival can be driven by specific antigen which persist *in-vivo* and provide continuous low level stimulations (161,282). Factors such as cross-reactive (31) stimulation or unknown homeostatic signals may additionally be involved

(214,215,287). The fact that the survival of memory cells is dependent on the presence of antigen, does not necessarily imply that the memory cells engage in a proliferative response to the antigen. Also the possibility that long-lived memory cells eventually become independent of the antigen can not be excluded (216), and unequivocal results regarding the necessity of antigen for survival of memory B cells remains elusive.

Current evidence suggests that memory cells have a more rapid turnover than virgin cells (218,276). Chromosomal lesions that preclude cell division were followed with time after irradiation [on the basis of earlier discovery (285)] in CD45RA (naive phenotype) and CD45RO (memory phenotype) T cells. The CD45RO with dicentric chromosomes disappeared from circulation within one year, while CD45RA cells survived for several years, without cell division. Other adoptive transfer experiments also support the notion that naive T cells and B cells in mice have a long life span (286). The life span of memory cells and hence the decay of the memory response may be related to the expression of molecules such as Bcl-2 (214,287), but the factors that influence Bcl-2 levels in memory cells are also not known. The pathways involved in memory cell generation are unclear. There is an emerging consensus that most specific lymphocytes involved in a response die of apoptosis (215).

The progression of T cells along the pathway to resting memory cells is dependent on the continued viability of the antigen selected cells and their resistance to activation induced cell death (AICD). The manner in which regulated cell death by apoptosis the generation of immunity and its subsidence is only beginning to be explored, although a number of the components of the cell death pathways have been identified. During their development and after

their functional activation most lymphocytes die, either because they fail to receive a survival signal or because they are actively killed. Recent experiments on Bcl-2 and Fas/APO-1 have provided evidence that these two death processes are subject to distinct genetic control.

Programmed Cell death/AICD/Apoptosis

The term programmed cell death (PCD) was coined by Lockshin and Williams (288) to describe the developmentally regulated death of specific larval muscles following the emergence of the adult moth. PCD originally implied that the death of cells occurred within a developmental context in response to a definable physiological stimulus. Developmental biologists use PCD to refer to the loss of specific cells in a temporally and spatially predictable manner that is induced by physiological signs as a normal component of the development of an organism. In contrast, immunologists use PCD to refer to any cell death that is dependent on *de novo* gene expression.

In 1972, Kerr, Currie and Wyllie proposed the term apoptosis to describe a common series of morphological changes that accompanied the death of cells from a wide variety of tissue sources (289). As cells undergo apoptosis, the surface membrane blebs and pinches off to produce blebs, referred to as 'apoptotic bodies' (290). Coincident with these morphological changes, the DNA within the nucleus is digested by an endogenous nuclease that cleaves chromatin between individual nucleosomes (291).

Cellular homeostasis in the lymphoid system is governed by the rates of cell development from progenitors, proliferation, differentiation and death. Apoptosis is the physiological death mechanism responsible for the removal of excess, obsolete or potentially dangerous cells (292,293,294,295). The effector mechanisms of apoptosis appear to be similar in all cell types and are remarkably conserved between species as

distantly related as the nematode *Caenorhabditis elegans* and the man (296,297). The fundamental importance of cell-death pathway is emphasised by its conservation throughout evolution (298). In fact, genetic studies of two invertebrates *Caenorhabditis elegans* and *Drosophila melanogaster* have provided the most insights in to the genes controlling apoptosis (299). PCD has been extensively studied in the live nematode *C. elegans* (300) in which the division and death of cells can be followed under the microscope. Many mutants of the death process have been identified and their molecular analyses have indicated that many gene products are involved in various aspects of cell death in *C. elegans*. A group of mutations, referred to as Ced (cell death abnormal) blocks loss of these cells (301). Systematic genetic studies of *C. elegans* have provided more direct evidence that proteases comprise the effector arm of the cell death pathway. Two genes *ced-3* and *ced-4* are required for all somatic cell deaths that occur during nematode development (302). Mutations of *ced-3* and *ced-4* abolish the apoptotic capability of cells that would otherwise be destined to die during *C. elegans* embryogenesis (303). The first mammalian homolog of CED-3 identified was interleukin 1 β (IL-1 β) converting enzyme (ICE) a cysteine protease responsible for the processing of pro-IL-1 β to the active cytokine (304). Subsequent studies have suggested that proteases related to ICE might be more important than ICE itself in the apoptotic mechanism (279).

The *C.elegans* model system has also provided insight into the negative regulators of the cell-death pathway. The *ced-9* gene antagonies the function of *ced-3* and *ced-4* by blocking cell death (305). Importantly, CED-9 is homologous to Bcl-2, the prototypic member of a growing family of cell-death regulators (306). First identified for its role in B cell malignancies, *bcl-2* when overexpressed inhibits cell death induced by a variety of apoptotic stimuli and in numerous cell types (214,307). The fact that *bcl-2* can substitute functionally for *ced-9* preventing cell death in the nematode, further

emphasizes the highly conserved nature of the cell-death machinery (306).

BCL-2 family

The BCL-2 family is comprised of both inhibitors of cell death, such as Bcl-2 and Bcl-XL, and promoters of cell death such as Bax and Bak (308,309,310). Importantly, BCL-2 family members can form homodimers and heterodimers (310,311). It was originally proposed that Bax (and presumably Bak) directly engages the cell-death machinery, whereas BCL-2 and BCL-XL merely antagonise this interaction (312). However, mutant forms of Bcl-XL failed to interact with Bax or Bak but still maintained death-inhibiting activity (313). Thus these findings support a model in which Bax and Bak promote cell death by sequestering Bcl-2 and Bcl-xL, preventing these negative regulators from binding and inhibiting their intended target.

During recent years, apoptosis although previously almost neglected, has become a major focus of investigation. The lymphoid system is particularly suitable for such studies as apoptosis plays an important role in development and maturation of both normal T and B lymphocytes and lymphoid malignancies. In the normal lymphoid system apoptosis occurs in primary lymphoid organs such as bone marrow, liver and thymus, and is used to eliminate useless precursor cells with non-rearranged or aberrantly rearranged, non-functional receptors. Furthermore, apoptosis is essential for deletion of autoreactive T cells in the thymus and is, therefore a mechanism to guarantee central self-tolerance (314,315,318). Staphylococcal enterotoxin (SE) superantigens delete in thymic organ culture, those thymocytes that express the corresponding SE-reactive TCR V β family (317). Moreover, endogenous superantigens such as those derived from mouse mammary tumor virus (MMTV) also induce,

intrathymic deletion (by apoptosis) of developing thymocytes expressing the reactive TCR V β elements (318).

In peripheral lymphoid organs, such as lymph nodes and spleen, a similar apoptotic deletion mechanism is operative in T and B cells. Peripheral deletion by apoptosis is another safeguard of the immune system to assure self-tolerance and to downregulate an excess immune response (319,331,332). Lymphocytes that escape this process might replenish the pool of cells that determine memory. The products of genes involved in the decision of lymphocytes to die by apoptosis or to survive are required for apoptosis, or induce or block apoptosis. Genes relevant for apoptosis might encode ligands and their receptors or a cascade of signaling molecules. Genes directly implicated in lymphoid apoptosis are p53 (320); cmyc (321); nur77 (322); pim-1 (323); several members of the bcl-2 family (307,309,310) and the gene encoding APO-1/Fas (CD95) (324). Within the lymphoid system most extensive studies have been done on APO-1/Fas and Bcl-2.

There are at least two types of cell death that are now recognised; necrosis and apoptosis (325). In necrosis, the cell undergoes irreversible swelling and lysis in response to a variety of signals which are primarily nonphysiological, the plasma membrane disrupts and then spills the intracellular contents into the environment. Apoptosis, by contrast, is inherently 'programmed' as part of the cellular processes allowing the cell to die in response to a variety of signals without a deleterious effect on surrounding cells.

A putative death stimulus will only trigger PCD in dependence of a determined context : the differentiation and activation states of the T cell, the presence of other costimulatory

signals, the metabolic environment, the presence of antigen etc. Thus, T cells fulfill a semiotic function in the sense that they continuously integrate stimuli arriving via multiple receptors that receive information from APC bystander cells, cell matrix, proteins, cytokines, hormones and the metabolic micro environment (152) and only a determined combination of signals will cause PCD.

Activation induced cell death (AICD)

Lymphocytes undergo activation in response to antigenic stimulation and as a consequence enter the cell cycle. The same antigenic signals that induces lymphocyte proliferation and other immune functions will also induce clonal deletion under some circumstances. This deletion occurs as a consequence of apoptosis - activation-induced cell death (AICD) understanding the fundamental mechanisms underlying this phenomenon is therefore crucial. In addition to its role in clonal deletion, another possible function for AICD may be to re-establish the immune repertoire following a strong response. A variety of conditions may give rise to AICD depending upon the status of the cells and or the nature of the activation signal.

Smith *et al* (326) first demonstrated the phenomenon of activation-induced apoptosis in thymic organ culture, where antibodies to the CD3-TCR complex induced apoptosis in CD4+CD8+ thymocytes. Similarly Shie *et al* (327) employed anti - TCR complex antibodies to induce apoptosis in immature thymocytes *in vivo*. Such observations were extended to TCR-transgenic thymocytes by Murphy *et al* (328) and Swat *et al* (329) who showed that exposure of such cells to their specific ligand induced apoptosis *in vivo* (328) or *in vitro* (329).

Ashwell *et al* (330) were among the first to discover that murine T-cell hybridomas undergo cell cycle arrest and cell death following activation. Their experiments demonstrated that death is induced by ligation of the TCR/CD3 complex with either antibodies or nominal antigens presented in the context of the appropriate MHC protein.

Several systems exist for observing AICD in mature T lymphocytes. *In-vivo*, administration of superantigens causes an expansion followed by a decline in the numbers of cells bearing the appropriate TCR V β for response to the antigen (319,331). This 'peripheral deletion' is associated with apoptosis in the responding cells at the peak of expansion (331,332,414). Presumably, this acts as a homeostatic mechanism to limit the expansion of lymphocytes specific for any one antigen and such a role for apoptosis in limiting hyperplasia has been observed in many systems (523).

T cells from asymptomatic HIV+ individuals have been observed to undergo apoptosis following activation *in vitro* (333,334). This observation supports the idea that AICD might have a central role in progression to AIDS (335).

Russel *et al* (336) showed that cells that are activated for a longer period, approximately 7 days, become sensitive to AICD. During primary activation, mature T cells from both human peripheral blood (337) and mouse lymphoid tissue (336,337) change from an AICD resistant to AICD-sensitive phenotype over a period of several days. This is different from the situation in the thymus, where the elimination of T cells appear to occur within hours of their first exposure to the deleting epitope (328). The complete molecular

basis for the T cells switch from an AICD resistant to an AICD-sensitive phenotype remains to be determined. Regulating the time needed to convert from a resistant to a sensitive phenotype is one means by which T cells can undergo primary activation without running the risk of elimination. It is only during the second encounter with antigen, that the responding T cells are at risk of deletion through AICD (338,339). During this second encounter the stimulus responsible for T cell death delivered via the TCR/CD3 also produces functional responses such as cytokine release or cytolysis of antigen-bearing target cells (339).

Fas-APO-1

Important breakthroughs in the understanding of AICD in mature T cells were the discovery that defects in the gene encoding Fas(CD95) were responsible for the lymphoproliferative disorder in *lpr* mice (340). Mature T cells from *lpr* mice were found to be resistant to AICD (341,342). The original description of Fas or APO-1 a member of tumor necrosis factor receptor family protein, indicated that it was expressed on activated, but not resting lymphocytes (343,345). Anti-Fas antibodies in particular trigger cell death in activated, but not resting human T lymphocytes (345). Triggering of Fas, necessary for death of the T cell is accomplished by Fas ligand (FasL) (345,346). Fasligand can be expressed on the same cell - cell-autonomous (Suicide) or on the other cell where contact-dependent (murder) pathway is elicited (339,346,347,348). It has been demonstrated that Fas can serve as a trigger for contact-dependent, cell-mediated cytotoxicity (349). There is evidence for both contact-dependent pathway and contact-independent pathways (350). The contact-dependent pathway is only operative at high cell densities and for optimal death, both the killer and the victim must be

simultaneously stimulated through their TCR(330). Thus, Fas behaves like a costimulator for death, as stimulation of either Fas or TCR/CD3 is insufficient to produce death, rather both must be stimulated concomitantly (339).

AICD of T cells is inhibited by a soluble Fas protein that binds to and functions as a competitive antagonist of FasL (346,347,348). Mouse strains carrying defect in the expression of Fas (lpr) or Fas ligand (gld) suffer from a generalized lymphoproliferative disease (351). Lpr and gld mice have a defect in the CD3/TCR dependent cell death pathway, suggesting that functional Fas/Fas-ligand expression is a prerequisite for apoptosis mediated via the CD3/TCR complex (352). Fas dependent death appears to be restricted to the peripheral T cell compartment, as thymocytes from both lpr and gld mice are sensitive to acute AICD and thymic deletion seems normal in these animals (353,354).

Studies on Fas showed that it plays a role in regulating a response (355,356). Fas is not important in death due to growth factor withdrawal (354). Russel (339,357), studying on the mechanism behind the two opposing responses of antigen stimulation, focussed on APC as a principal regulator. Using TCR-transgenic mice he showed that costimulator molecules on APCs can open the window between the threshold stimulus for cytokine production and T-cell death. By selectively transfecting B7.1 he showed the antigen-dose threshold for cytokine production by Th1 cells lowered, without affecting the threshold dose for stimulating Fas-dependent, Th1 death. Suggesting, that a specific state of activation is required for sensitivity to AICD, perhaps including a specific point in the cell cycle (358).

Interestingly, stimulation through Fas does not always induce a death signal: indeed, ligation of Fas or freshly isolated T cells has been demonstrated to costimulate cellular activation and proliferation (355,356,359). Why Fas transduces a costimulatory signal in resting T cells but an apoptotic signal in previously activated T cells is unclear. There is some evidence that expression of Bcl-2 (215) or other members of the Bcl-2 family, may result in the resistance or sensitivity of T cells to apoptosis (309,310,360,361). The expression of Fas and FasL has also been evaluated on Th cell subsets, focusing on expression of these molecules by Th1 and Th2 cells. Th1 clones are highly sensitive to AICD whereas Th2 clones are relatively resistant (352). Both Th1 and Th2 effectors express Fas and FasL, but Th2 effectors also express FAP-1 a Fas-associated phosphatase implicated in interfering with Fas-FasL mediated apoptosis (362) which may account for the lack of Th2-susceptibility to AICD.

Studies On B-Cell

Engagement of CD40 on B cells induces the expression of Fas, and makes B cells sensitive to Fas-mediated apoptosis (363,364). By contrast, cross linking of the antigen receptor on B cells induces a state of resistance to Fas-mediated apoptosis (365). The expression of Fas on B cells, and sensitivity to Fas-mediated death are tightly regulated processes. For B cells, Fas mediated apoptosis regulates the net result of interactions with helper T cells in extrafollicular regions of lymphoid tissues. Although Fas is expressed on germinal centre B cells (366), there is little evidence that it is involved in the selection of high-affinity B cells in germinal centres (356). Fas-mediated death is also important for the elimination of self-reactive B cells (367).

In a search of more proximal component of Fas a new protein has been identified called FADD for Fas-associated death domain (368). Overexpression of FADD in cells is sufficient to induce apoptosis. Binding of FasL to Fas may result in receptor aggregation and the association of FADD and other proteins with Fas. It is also likely that Fas-mediated apoptosis is negatively regulated at multiple points. For instance the cytoplasmic end of Fas contains a sequence that blocks apoptosis (369) this 'salvation domain' associates with a tyrosine phosphatase that may function to dephosphorylate Fas and/or associated proteins (362).

Antigen specific non-responsiveness

Recognition of antigen does not always lead to activation and differentiation in to effector response, instead sometimes, it leads to a state of non responsiveness. Effective T cell immunity is dependent on the clonal expansion of the small population of cells that express antigen receptors specific for the immunogen (370). Immunologists have known for over 30 years however, that certain forms of antigen administration not only fail to elicit a T cell immune response but also render the host unable to respond to a subsequent challenge with an immunogeneic form of the same antigen (371,372,373,426).

State of antigen specific non responsiveness can be due to two different mechanisms,

Tolerance - in this, the host is unable to mount a response to a specific antigen because the relevant T or B cell clones are either deleted or rendered non responsive.

Suppression - in this, the T or B cell clones recognizing a given antigen are normal and responsive however, they are prevented from responding to an antigen by other cells. The phenomenon is called "suppression" and the cells responsible for it the "suppressor cells".

Tolerance

Immunological tolerance is the most fundamental property of the immune system that nature employs in avoiding the horrors of autotoxicus and regulating the level of responsiveness to extrinsic antigen per se. The word tolerance was first applied to this field by Owen (326) in his classical studies on binovular twin cattle sharing common placenta. Burnett and Fenner (375), later predicted that an antigen introduced in to the body during embryonic life before the immune apparatus had developed, would be mistaken for self and that it would not evoke response then or if encountered later in life.

Tolerance is learned and is specific. This phenomenon has been repeatedly confirmed and has led to a definition of tolerance as a "specific unresponsive state induced by prior exposure to an antigen".

Self and foreign antigens are a motley lot : Both are encountered by the immune system in a broad range of concentration, physiological forms and tissue locations. Understanding how self antigens tend to tolerize while foreign antigen tend to immunize is at the heart of the problem of tolerance. The development of immunity or tolerance both of which involve specific recognition of antigen by antigen reactive T or B cells, needs to be carefully regulated since an inappropriate response whether it be immunity to self antigens or tolerance to a potential pathogen can have serious and possibly life-threatening consequences.

Tolerance can be generated and maintained in a variety of ways. Great advances have been made in recent years in formally demonstrating that some of the theories proposed many years ago are fundamentally correct.

Examples of tolerance induction.

Several different experimental protocols have been employed to study tolerance. In the 1960's there was considerable interest in the idea that prolonged exposure of lymphocytes to antigen can lead to exhaustion (376). The first direct evidence for this idea has come from the finding that the V β -specific elimination of T cells at the end of the primary response to superantigen can be nearly complete and lead functional tolerance (319,331). High doses of antigen can paradoxically suppress immune responses in adult animals and there are many examples of tolerance developing , given large doses of antigen in various forms (377,378).

T cell unresponsiveness was induced in CD4⁺ helper T cell clones (379,380,381) as a consequence of their stimulation with antigen-MHC complexes in the absence of costimulatory signals provided by APC's. In most such experiments, the tolerance could be reversed by either bystander APC's expressing B7 or by IL-2 (122,382,383). The capacity of unresponsive T cells to produce IL-2 or to proliferate was found to be blunted, regardless of the level of antigen presentation and costimulation provided by APC's during rechallenge (119). It was these observations that led to the proposal of a 'two-signal' model of T cell activation that holds that signals sent to the T cells through their antigen receptor and costimulatory receptors promote T-cell responsiveness, whereas TCR signalling alone induces

subsequent functional unresponsiveness (122,123,128). Recent work has demonstrated that CD28 is an important costimulatory receptor on T cells: CD28 occupancy by its APC ligands B7.1 or B7.2 or by anti-CD28 antibodies greatly augments the amount of lymphokine that the T cell makes in response to TCR stimulation (115,138) and prevents the induction of T-cell unresponsiveness (122). Tolerance induction by anti-CD3 antibodies also seems to occur by the same mechanism, i.e. the antibodies possibly substitute the signal for MHC-peptide complex; the costimulatory signal being absent, results in to tolerance.

Stimulation of T cell clones with peptide antigens presented by other T cells, or stimulation of ex-vivo T cells by allo-MHC class II presented by activated T cells, is yet another example of tolerance induction (384,385,386). The class II expressing human activated T cells do express B7 (387), hence mechanism of tolerance induction in this experimental protocol would be different. Since activated T cells express Fas ligand, the tolerance induction could be mediated by Fas interaction (388). However, in some of the experiments of tolerance secondary to T cell antigen presentation, tolerance has been reversed by IL-2 (382,383). Perhaps exogenous IL-2 stimulates Bcl-2 expression, thus protecting the cells from Fas mediated tolerance (307,389). T cells that present antigen, also express CTLA-4, the second surface receptor capable of interacting with B7. CTLA-4 : B7 interaction has also been alleged to be suppressive to the T cells recognizing the antigen presented by CTLA-4 expressing T cells (147,148,390).

Experimental protocols, to highlight the role for co-stimulation of B cells, in prevention of tolerance have also been described. Antigen activated B cells can be rescued from tolerization

by helper T cells; in this interaction between B cell CD40 and T cell CD40 ligand (gp39) plays an important role. Further, engagement of CD40 on B cells induces the expression of Fas and makes B cells sensitive to Fas mediated death (363,364).

Allen *et al* (391,392) have reported a novel method for inducing T cell unresponsiveness in-vitro. They demonstrated that T cells stimulated with an altered peptide ligand (APL) analogue of an antigen, induce tolerance. Every encounter of T cells with such antigenic analogues, inevitably results in the T cells losing their capacity to produce IL-2; if growth factor production and proliferation do not accompany the initial exposure to antigen, the functional tolerance persists in the T cell.

Central tolerance due to clonal deletion.

More than three decades ago Burnett (366) and Lederberg (393) proposed physical deletion of self-reactive lymphocytes as a mechanism by which self tolerance is acquired. Later the phenomenon was described in normal mice by showing deletion of T cells specific expression of V β +TCR in conjunction with endogenously encoded viral superantigens (318,403). This concept also referred to as central tolerance was solidified in transgenic mice in which genes that encode a TCR capable of recognizing specific self antigens had been introduced in to the germline, under conditions where the relevant antigen could be demonstrated to be present in thymus. As a consequence, T cells with the transgenic TCR were found to be absent in these mice (394).

Tolerance induction in immature B and T cells occurs in the primary lymphoid organs bone marrow and thymus during the

process of maturation. Many of the body's self antigens are present in such abundance that they are easily presented within the thymus to developing T cells. Tolerance to such antigens has been shown to involve deletion of the reactive clone (395). As repeatedly shown in transgenic model system, antigens present in that critical developmental period induce tolerance, irrespective of their genetic origin.

Various mechanisms have been described that dictate the recognition of the antigen in the thymus (395,396,397). Regardless of which of these mechanisms, it is clear that one requirement for thymic deletion that must be met is that antigenic peptides derived from proteins are presented at a sufficiently high density in the thymus to allow a threshold of receptor occupancy to be reached. Variables such as antigenic peptide concentration, density of the MHC molecules presenting those peptides and affinity of the TCR for the peptide/MHC ligand, together dictate whether a certain threshold of receptor occupancy is reached for deletion (101,401,402). Participation of the coreceptors CD4 and CD8 (398); co-stimulatory molecules CD28 and gp39 (399) also affect thymic deletion by regulating expression of B7 on thymic APC's (400).

Peripheral tolerance

Deletion of self-reactive T cells in the thymus is probably the most important mechanism for the establishment of tolerance (318,401,402,403). In addition to this central tolerance, there is also the need for peripheral tolerance mechanisms for cells having escaped thymic deletion. T cells have developed multiple ways in which tolerance can be achieved in the periphery, which has been attributed to two main mechanisms. The potentially reactive cells are eliminated -

clonal deletion or they are functionally silenced or inactivated- clonal anergy.

Amongst various experimental protocols described earlier, anergy or deletion or both play the part in the mechanism of tolerance. Experiments with superantigen mediated interaction display both anergy and deletion as the causes of non-responsiveness. Prolonged persistence of anergy eventually leads to clonal loss.

Anergy

A minimal definition of anergy is the situation where T cells that can recognize a particular antigen are present, but fail to respond when challenged with that antigen. Anergic lymphocytes from immunized or infected animals are immunocompetent cells, differentiated or reversibly induced in to non functional states. Anergic cells excitable (404) and programmable (405) and it is reasonable to postulate that they are immunologically active (406). Many demonstrations of anergy are presented as a failure of T cells to proliferate or produce IL-2 when stimulated *in-vitro* either with antigen, but without costimulation or with certain antibodies to the TCR (407,408). The term anergy is rather a misnomer because anergic T and B cells seem to retain some of their functionality and can be stimulated out of their anergic states.

Lamb et al (379) and Jenkins and Schwartz (408) showed that cloned Th1 population of either human or murine origin can be functionally paralyzed. The paralyzed cells were rescued to functionally active state- by delivery of costimulatory signals delivered through B7-CD28 pathway (122). Anergic cells can also

recover through rounds of IL-2 driven proliferation (383,384). This feature leads to the possibility that an extremely strong stimulus may actually rescue paralyzed cells through production of lymphokine. Such rescue would result in their recruitment in immune response. Anergic cells can also recover reactivity when they are parked for a short time outside the spleen of specific antigenic stimulation (409,410).

Features of anergic cell

Anergy primarily affects the capacity of T cells to produce IL-2, while the production of other cytokines IL-3, IL-4 or IFN- γ blastogenesis or IL-2 receptor expression are much less diminished (407). Studies have shown that anergic T lymphocyte clones have altered inositol phosphate, Calcium and tyrosine kinase signalling pathway (411,412). Although anergised T cell clones retain their cytokine activity (413). The block in IL-2 production that develops in non-responsive Th1 clones is not indicative of a global defect in lymphokine production (408). It has also been observed, that in T-cell clones that are capable of producing both IL-2 and IL-4 - Th0 (415,416) and more recently in Th2 clones (392), the IL-4 gene remains inducible even after the induction of clonal anergy. In these cells, autocrine growth is blocked because the T cells can not respond to the IL-4 produced by them. Functional tolerance has been shown with downregulation of TCR's and coreceptors (417,418,419,420). Persistent antigen exposure has experimentally shown in transgenic mice to result in to downregulation of coreceptor expression and subsequent non-responsiveness (420). Phenomenon labelled as "receptor desensitization" has also been described in-vivo when T cells undergo repeated stimulation by normal APC within a short period of time (422,423,424). T cells fail to divide in response to the

repeated stimulus despite normal IL-2 production. T cell paralysis or nonresponsiveness may take various forms ranging from down regulation of coreceptor and TCR, to inability to respond in ways yet to be defined at the molecular level and which are flexible, dynamic and reversible.

Deletion

Clonal deletion in the thymus is the principle method for purging the immune system of autoreactive T cells. Clonal elimination also operates in the peripheral lymphoid tissues and affects mature T cells responding to foreign antigens (425). Physiological cell death occurs in activated B and T lymphocytes during immune responses and as in immature cells, it can result in either from the absence of a survival signal or from the activation of programs leading to cell death - apoptosis represents a critical element in the maintenance of homeostasis within the immune system.

Interactions between Fas ligand and Fas play an obligatory role in apoptosis of T cells that are triggered by activation in so called activation induced cell death (AICD) (345,346). If activated T cells are stimulated via their antigen receptors and produce IL-2, they co-express Fas and Fas L and rapidly undergo apoptosis (356). Expression of Fas is tightly regulated and requires continuous stimulation. For long it was known that high doses of antigen can paradoxically suppress immune responses in adult animals (427,428,429). Studies indicate that the stronger the initial proliferative response to antigen the more vulnerable T cells are to apoptosis by antigen re-engagement (338). At least for cell-bound superantigen, the extent of clonal elimination of T cells correlates

closely with the intensity of the initial phase of T cell proliferation (339). Thus, a strong proliferative response is followed by exhaustive T cell elimination and tolerance.

Several important issues about the role of Fas in T-cell apoptosis are becoming apparent. T cells die upon repeated activation or as a result of inadequate stimulation, Fas mediates the former (388,430). Apoptosis as a result of inadequate stimulation, which may be more appropriately called PCD is prevented by CD28-mediated costimulation, growth factors such as IL-2 and by activation of survival associated genes such as bcl-2 and bcl-xL (388,431). In contrast, Fas mediated AICD is not blocked by costimulation, actually augmented by IL-2 and not counteracted by bcl-2 or bcl-xL (388,430,432,433).

Although immature thymocytes express Fas and are susceptible to Fas mediated killing, negative selection of developing thymocytes does not require an intact Fas-Fas L system (354). The importance of Fas - mediated apoptosis in mature T cell death is supported by the finding that mature T cells from Fas defective -lpr and Fas L defective -gld mice are resistant to AICD both in-vitro and in-vivo (341,342), whereas thymocytes from both these mice are sensitive to acute activation induced death (354).

There are other interactions involved in cell death other than Fas -Fas L. In some of the CD8+ T cells perhaps even some CD4+ cells AICD has been shown to be by TNF-TNF receptor interaction (439). CTLA-4 mediated signal transduction has also been reported to induce cell death in previously activated T cells. Studies have suggested that the negative regulatory effect of CTLA-4 on immune responses reflects a block in IL-2 production and a block of

progression through cell cycle (435). Cross-linking CTLA-4 blocks response 24h after activation (435) whereas Fas does not become functional until 3 days after activation (437,438).

Variables that determine whether peripheral elimination proceeds efficiently, include extent of TCR occupancy, affinity of the antigenic peptide for MHC and affinity of the TCR for its MHC-peptide complexes.

Both Fas-induced T cell AID and anergy result in a reduced responses. An important distinction between them is, however, that the T cells from Fas-dependent AID produce IL-2 (388) whereas in the anergic response the failure to proliferate results from a block in cytokine production (122). It is important to distinguish between these two mechanisms in studying the down-regulation of T cell responses. Anergy may play a more central role in regulating the primary activation of T cells, whereas AID may be more important in limiting the further expansion of activated populations.

The silencing of T cells upon persistent engagement of their antigen receptors in the periphery may thus represent a continuous process, ranging from activation to unresponsiveness to deletion, with signal strength and exposure time together determining the outcome.

Taken together functional silencing may reflect different mechanisms/pathways and levels of tolerance that can be achieved either by activation of T cells with antigen presented by non-professional APC or by activation of T cells with high doses of either self (439) or foreign antigens (440,441). At one end of this spectrum anergy as defined by the selective inhibition of the IL-2 pathway may

reflect an intermediate state of functional T-cell silencing somewhere on the way between normal reactivity and exhaustion which is final stage of functional silencing which precedes cell death (419,442).

Role of tolerance in immune homeostasis.

T lymphocyte proliferation must be tightly regulated to protect against the development of in-appropriate immune responses to self-antigens or exaggerated pathological responses to foreign antigens. Given the importance of avoiding uncontrolled autoimmunity, immune system uses a repertoire purging mechanism with a suppressor system that acts as a fail-safe device. Given the immunological consequences that can result from continued antibody production in the face of persisting antigen, immune system utilizes a trick developed primarily for self-recognition to limit the immune attack against antigen. Deletion of autoreactive T-cell clones during maturation in the thymus is the first and the main mechanism to avoid self-recognition. Tolerance to alloantigens can be induced at all stages of development of the immune system although inducing tolerance in adults where the immune system is already mature is arguably the most difficult situation in which to achieve tolerance. Many factors influence whether tolerance to alloantigens can be induced and a number of mechanisms are implicated in both the induction and maintenance of the tolerant state. Some lymphocytes with self-reactivity ignore the self antigen in question because of low-affinity. Lack of effective presentation and appropriate T cell activation signals when T cells encounter self antigen maintain tolerance induction. Such lymphocytes form the substrates of autoimmune phenomena should they be activated following changed operational circumstances.

High antigen density and a lack of costimulatory molecules on tissue APC favours T cell death as well as cytokine production (339). Cytokine deviation has also been cited as examples of tolerance induction (443). Specific state of activation is required for sensitivity to AID perhaps including a specific point in cell cycle (358). In any event, negative control of responses through Fas-dependent AID may require multiple exposure to antigen to lower the number of responding cells below a critical threshold.

Tolerance to self antigens can be broken in several ways. Since most normal individuals can be induced to develop auto-reactivity, but few develop autoimmune disease. It is clear that clonal deletion of all auto-reactive cells does not occur. In some cases, auto-reactive T cells do not cause pathology because they do not see self antigen-tolerance through ignorance. In other, the T cells bear low affinity receptors and require high antigen concentration and costimulation to become activated. Provision of T-cell help or costimulation can initiate T or B cell auto-reactive responses. Infectious agents may participate in the induction of autoimmune disease through the process of mimicry, or through other effects including tissue damage and release of sequestered antigen, availability of cryptic self-determinants through increased MHC expression, ectopic expression of molecules redistribution of intracellular molecules to the cell surface, upregulation or shift in the spectrum of cytokine production and bystander activation of T cells. Slow and persistent release of self antigen due to tissue damage, in fact enhances immunity instead of tolerance.

Russel (339) suggested that the tolerance promoted by Fas-dependent AID is regional, rather than global. Potentially reactive T cell precursors in lymphoid tissues distant from the region of

antigens source would not be activated and therefore not sensitive to elimination. Finally, it must be stressed that autoimmune disease is multifactorial and the presence or absence of a functional, Fas dependent AID system is simply insufficient to predict sensitivity to all autoimmune responses.

Field and Loh (444) reported transgenic system and Russel (339) described an experimental evidence supporting a model that proposes that TCR engagement of activated T cells has opposing effects on the capacity of that cell for future inflammation. Failure to trigger Fas-dependent AID allows continued activation and the production of additional cells to increase inflammation. Triggering the Fas -dependent AID pathway results in the death of the cell and its elimination from participation in future inflammatory responses. If triggering Fas-dependent AID were an efficient process, it would be difficult for the immune system to mount a protective response to pathogens. Thus, it is necessary that positive and negative results be carefully balanced.

Self-recognition is not absolute. Some autoantibody production is consistent with health and may even be physiologically useful for the disposal of dead cells or cellular debris. The important thing is to limit the amount, affinity and duration of autoantibody formation, particularly after tissue damage, and against cell-surface macromolecules.

To ensure this controlled situations, the immune system makes use of every regulatory processs it possesses. Afferent-limb-deficiencies that is, failure of a self antigen effectively to engage the interest of APC- probably play a role in the overall end-result of self tolerance, and events which prompt unusual degrees of

autophagocytosis or unusual presentation of cell-surface constituents may play some part in the spectrum of autoimmunity.

Immunesuppression

Although clonal deletion of specific T cells in the thymus appears to be the major developmental mechanism for generating tolerance neonatally, there is increasing evidence that unresponsiveness can be maintained in adult individuals despite the existence of antigen reactive T cell clonotypes in the periphery. Immune-suppression is a frequently encountered feature of chronic infection. In the past the term, was commonly used to describe the reduction in immune responsiveness associated with the appearance of CD8+ suppressors T cells (37,40,41,42,446,448).

Classic experiments by Gershon and Kondo (445) in 1971 showed that adoptively transferred spleen cells were able to specifically inhibit humoral immunity in vivo. Cells mediating this effect were shown to be thymus-derived and antigen-specific and were later referred to as suppressors T cells (Ts) (446). In vitro studies showed that Ts cells also specifically down regulate or interfere with antigen induced T cell proliferative responses (447). The demonstration that Ts cells can specifically render other lymphocytes unresponsive to antigen suggested an alternative mechanism, in addition to tolerance for maintaining non-responsive state to an antigen and raised the possibility that Ts cells play an important role in immune regulation (40,449).

For many years the idea that suppressor cells were able to control immune responses and could be responsible for peripheral tolerance received widespread credence (37,40,42,449). This idea later

fell into disrepute, largely because it has been more or less impossible to isolate and fully characterize the cells supposedly responsible for suppression. Further, in several of the models described workers have looked for, but failed to find, evidence of suppressor cells. Attempts for cloning also failed, as they were observed to have one or more T cell functions other than suppression (38). However, Ts cells have been defined almost exclusively by their biological activity. Consequently, a variety of T-cell phenotypes have been associated with unresponsiveness *in vitro*, in ways very difficult to assimilate in a coherent pattern (36). Both CD4 and CD8 subsets in various systems reportedly are involved in immune response suppression (34,450,451). Some workers favored anti-idiotypic mechanisms, whereby suppressor T cells recognize the relevant idiotypic determinants on alloreactive T cells by means of their antigen-specific receptors and suppress their function (452). Others favored a veto mechanism, whereby suppressor cells bearing the tolerated alloantigen in their membranes inactivate any alloreactive cells which recognize them (453,454). Many T-cell derived soluble antigen-specific mediators referred to as suppressor T cell factors or Ts factors, were believed to mediate interactions among cells in the pathways (455,456,457). These factors variably displayed MHC the controversial IJ or Igh-linked restricted activity (458). Despite the profusion of suppressor factors, none have been adequately characterized in biochemical or genetic terms. Lack of unique surface markers which permitted identification of distinct cell types, and difficulties encountered in cloning the various Ts cell populations further compounded the problem. Skepticism concerning Ts cells and their physiological role began to escalate in the early 1980's and culminated with some questioning their very existence (43,44,459).

Despite a prevailing pessimistic attitude in the scientific community, significant progress has been made in our understanding of Ts cell phenomenology (36,43,45,460). There are too many well-documented experimental examples of suppression *in vivo* and are believed to play a role in immune-regulation also.

Studies in a number of systems provide evidence for an important role for Ts cell in preventing autoimmunity such as EAE (461). Immunesuppression is clearly demonstrated in the chronic human infectious disease - leprosy (34,35,37). Patients fail to develop detectable cell-mediated immunity to antigens of *M.leprae*. Several laboratories have observed antigen-induced suppression of CD4+ T cell responses to antigens in vitro mediated by CD8+ T cells from lepramatous patients (34). Further studies revealed that CD8+ Ts cells were able to suppress CD4+ T cells in a MHC class II restricted fashion (462).

Antigen specific immune suppression, as a phenomenon can be explained without proposing a specific class of professional "suppressor cells". Activated T cells can suppress other T cells in vitro simply by competing for available growth factor (463). Ts cells can also inhibit the activity of another cell such as other T cells, B6 cells and macrophages in a variety of ways including cytotoxicity, release of inhibitory cytokines such as IFN- γ , IL-10 or TGF- β and possibly by cell-cell contact followed by delivery of a noncytolytic negative signal to the target cell (464,465,466,467,468). Suppression can affect precursor clones by competition for antigenic peptide or for growth factor by irrelevant clones (46).

Despite the controversial issue of Ts cloning several laboratories have claimed success (469). Stable CD8+ T clones were

established by periodically stimulating with antigen and addition of IL-2 containing medium. They were shown to be classical effector type Ts of CD8+ phenotype as they had constant and definitive suppressor function in antigen-induced T cell proliferation and specific antibody response against T cell dependent antigens without detectable cytotoxic activity against both APC and Th cells.

Thus the question of T suppressors cells with all the controversies remain an ever challenging issue and much more progress is needed to understand the phenomenon completely.

Significance of the study

Effector phase and memory of immunity arise through complex regulatory processes of adoptive response to foreign antigen. The effectiveness of an immune response is dependent on how many effector cells are generated on how long they persist and whether they secrete the right cytokines and carry out the essential effector functions which are appropriate for the particular antigenic challenge. Long-term immunity is dependent on whether the highest-affinity cells are selected for conversion to resting memory and on how many memory cells are generated. Efforts to understand the complex regulation of immune response to an antigen have given way to enormous advances in the study of different signaling pathways and the interplay between them. We clearly have big gaps in our understanding of how recognition is translated into response and how so many different signals can be integrated to affect the fate of immuno competent T and B cells in so many different ways. New understanding of the pathways of death and anergy as well as positive responses will also be critical in building an accurate picture of the overall course of the immune response.

In this present study the following two important factors that decide the outcome of immunisations in terms of size of effector and memory response of CD4⁺ T helper cells have been investigated.

- Events of activation that induce clonal loss and thus reduce the size of memory and effector pool.
- Factors leading to activation of functionally irrelevant CD8⁺T cells capable of abrogating or reducing the size of CD4⁺ Tn cell memory and effector response by competing for antigen and/or lymphokines required for growth and differentiation.
- Events affecting activation that lead to clonal loss.

Both immunity and nonresponsiveness due to tolerance or death may represent different outcomes of antigen induced activation. To investigate some of the events that can lead to AICD, we have employed an experimental model of chronic graft versus host disease (GVHD) in which profound chronic donor CD4⁺ T cell activation is triggered by the alloMHC-class II antigen of host cells, this in turn has been shown to affect the outcome of immunisation to unrelated antigen.

In order to understand the relevance of the use of chronic GVHD as an experimental model system a brief information on chronic GVHD needs to be reviewed.

Experimental Model of Chronic Graft Vs Host Disease

In 1953, the term Graft vs Host reaction was introduced into the literature simultaneously by Simonsen and Dempster who studied kidney allografts in dogs and found that the cells reacting against host alloantigen were of graft origin (470,471). Since then GVH reaction has become a remarkable experimental system to study various abnormalities and over the years it has contributed to many immunological concepts (55,56,57,479).

When parental strain lymphocytes are injected into adult F1 hybrid (i.e. Semi allogeneic) mice, the recipients are genetically tolerant of parental strain donor cells but the grafted T lymphocytes on the other hand react against allogeneic histocompatibility antigen of the host and thus induce a spectrum of pathological alterations and profound immunologic consequences for the F1 host (56). The generation of these immunologic changes particularly in a subject of experimental animals showing a chronic GVH reaction depend on the presence of an intact lymphohemopoietic system of the host and vary depending on factors such as donor and host MHC differences (56,473,482), donor cell inoculum (474) and donor T-cell phenotype (475,476,480) most of the pathological alterations induced by the GVH reaction in non irradiated F1 mice also occurs in GVHD of man (477) and animals (478).

In non-irradiated F1 recipients there are two forms of GVHD (56). The injection of donor cells that differ from the F1 and both class I and class II MHC loci results, in what has been termed as lethal, acute or immunosuppressive GVH reaction and is characterized by anemia, hypogammaglobulinemia, depressed *in-vitro* T cell function,

atrophy of lymphoid tissue and eventual mortality generally thought to be due to an increased susceptibility to infection (56,482).

The other form called chronic GVHD has stimulatory pathology. There is a persistent lymphoid hyperplasia of mainly the B lymphocytes of host origin that leads to hypergammaglobulinemia and the formation of autoantibodies characteristic of systemic lupus erythematosus (SLE) such as antibodies against nuclear antigens, double stranded DNA (ds DNA) erythrocytes and thymocytes (56,474,480).

Murine chronic GVHD model has helped to study defects behind the human autoimmune disorders (57). Studies in this experimental system suggested that cytotoxic T lymphocytes play a critical role in the prevention of autoimmunity (475,480). Critical for production of chronic GVHD is the absence or low number of CD8⁺⁺T cells in the parental donor cell suspension employed for inducing GVHD. A normal number of CD8⁺T cells transferred induces acute GVHD, whereas low number or total absence leads to chronic GVHD (476,481).

Early in the history of AIDS epidemic, Shearer and colleagues (57,479,485) drew attention to the similarities between chronic GVHD and AIDS. Classical changes in the early phase of HIV infection are all features of characteristic chronic GVHD namely loss of weight, lymphadenopathy with B cell hyperplasia, polyclonal B cell activation and random generation of autoantibodies.

In the early stage of the disease, animal harbouring chronic GVHD show inability to mount self MHC restricted CD4⁺T cell responses to foreign antigens (57). Yet the cells respond to polyclonal

mitogen normally. In the late stages of chronic GVHD there is immune deficiency with loss of CD4+T cells (485). These features are also seen in chronic asymptomatic HIV carriers (479,483,484,485,486,487). Eventual CD4+T cell loss in HIV infection is well known several hypothesis, based on *in-vitro* experimental studies, to explain the CD4+T cell loss, have been proposed. A common theme in all these hypotheses is the induction of apoptosis by CD3- TCR signaling (339).

Several investigations have suggested chronic GVHD to be an experimental model mimicking immunological changes seen in chronic HIV infection (480,483,489). Most interesting feature shared by the two diseases is the pathogenomic progressive immune deficiency coupled with features of polyclonal T cell stimulation (480,482,485,486). The exact pathogenesis of this is still not clear. An investigation in this area employing the chronic GVHD model is likely to provide new insights in the area of activation induced CD4+T cell death in general and in HIV infection in particular.

Factors Leading To Activation Of Functionally Irrelevant CD8 + T Cells Which Abrogate The Response Mediated By CD4 + T Cells.

The second issue affecting the outcome of immunization namely activation of CD8 + T cell clones capable of competing with CD4 + Th cells, was studied employing an experimental murine model of CD8 + T cell mediated suppression reported by us earlier (46).

Over the past few years our group has been studying route related variation in immune response of mice immunized with *M. vaccae*. Immunization with *M.vaccacae* by intradermal (i.d.) or

intraperitoneal (i.p.) route results into two different outcomes. Mice immunized by i.d. route mount a good cell mediated immunity characterized by DTH and lymphoproliferative responses whereas in the i.p. immunized mice the responses were poor (4,41). Further it was shown that i.p. immunization induced antigen specific immune suppression which was mediated by the expansion of CD8 + Ts cells (41). In spite of the strict regulatory pathways in processing and presentation of Class I restricted CD8 + T cells, the *in-vitro* suppression mediated by these CD8 + Ts cells was blocked by anti IE antibodies suggesting that these cells recognized the antigen presented by IE (489).

Further investigation on these unexpected observations of i.p. primed CD8 + Ts showed that they could compete for growth factor IL-2 and thus suppressed the response of simultaneously primed CD4 + Th cells (46).

So far the observations, did not lead us to any specific conclusion as to why competing CD8 + T cell clones recognized antigen on aberrant MHC. T suppressors have been a mystery for the Immunologists for quiet a few years. It is unlikely to be coincidence that in almost all studies of CD8 + T cell mediated suppression in the mouse, antigen specific Ts cells have been II restricted (46,460,489,490,491). All of the CD8 + Ts cell clones in leprosy (460,490) and suppressor cells of a patient with Schistosomias (491) were found to be HLA-DQ restricted. Furthermore, a number of human autoimmune diseases are linked to HLA-DQ and mice lacking IE have a high incidence of susceptibility to induced autoimmunity (460). Even in our experimental system, we did observe that IE negative strain mounted a DTH response on i.p. immunization, suggesting IE restriction of Ts cells (489). But *in vitro*

studies showed that i.p. immunization did expand CD8 + T cells which recognized antigen presented by IA, IE and also on allogeneic IE (46). In the light of this observation the obvious question arises whether antigen recognition by CD8 + T cells is truly class II restricted or is it class II dependent.

Class II dependent recognition and even recognition on allogeneic MHC - is a characteristic of recognition of superantigen (58,492,493,494), which tempted us to hypothesize that superantigen interactions are involved in the recognition and expansion of CD8+ Ts cells. We do not know if the Mycobacterial antigen employed in the experimental model i.e. 65kDa antigen of *M.vaccae* is truly a superantigen.

The fact that IE negative strain did show the expansion of CD8+ Ts on ip immunization suggested that some unknown IE dependent factor was necessary. Peritoneal macrophages and B cells the, ACP's in the peritoneum carry mls expressing superantigen. Also i.d. immunization does not generate suppressors and APCs in the skin, the Langerhans cells and the dermal dendritic cells do not express superantigen of the mtv loci (495). These facts further strengthened our speculation that superantigen mediated interactions are involved in the activation and expansion of CD8 + Ts cells which recognize antigen on MHC II IE.

Janeway proposed a coligand hypothesis which state that all TCR-MHC interactions are stabilised by a coligand (496,497). He proposed a model for the action of superantigens, which proposes that superantigens are recognized as intact proteins that bind to the lateral face of the MHC class II molecule and the lateral face of the V β domain of the T cell receptor (498).

It is now widely accepted that the mls genes and other genes with similar properties are encoded in the mouse genome as mouse mammary tumor virus integrants. In addition, exogenous mouse mammary tumor virus itself encodes superantigens that can be transmitted horizontally through milk (513). In the light of all these facts, we reasoned that viral superantigen of MMTV origin acts as a coligand and facilitates the recognition of our experimental system bacterial antigen recognized by CD8 + Ts cells and thus bringing about suppression of the response of simultaneously primed CD4 + Th cells.

Thus, in this present study we are investigating two events which affect the size of the effector and memory response :

- I. Events of activation that induce clonal loss and thus reduce the size of memory and effector pool and,
- II. Factors leading to activation of functionally irrelevant CD8 + T cells which abrogate the response mediated by CD4 + Th cells.

Thus, by employing the two experimental model systems described in the last sections which seem to affect the size of an activated T cell clone, the evolution of memory and effector CD4 + T cells response after immunization was studied.

AIMS AND OBJECTIVES

⇒ To establish an experimental chronic Graft Virus Host Disease (GVHD) in mice.

⇒ Study generation of effectors and memory response of CD4 + T helper cells in them.

⇒ Study factors affecting the size of suppressor CD8 + T cell response.

MATERIALS AND METHODS



Animals

Mice- 8 - 10 week old C57 BL/6 (B6) and C3H mice obtained from National Institute of Health , USA and maintained at Cancer Research Institute , Mumbai were used in the study.

(B6 Female X C3H male) F1 and (C3H Female X B6 Male) F1 were raised and maintained in the animal colony of Haffkine Institute. Prior to immunization all mice were tested for delayed type hypersensitivity (DTH) response to the sonicate of *M.vaccacae* only those that showed no response were used in the experiments.

Rabbits- Adults Albino Rabbits weighing 1.5 - 2 kg were purchased from Haffkine Biopharmaceutical Corporation Limited, Mumbai; were used for raising antisera.

Microorganism

Mycobacterium *M. vacccae* was kindly supplied by Dr. J. L . Stanford of Middlesex Hospital , London. The strain was maintained on Lowenstein Jensens (L J) slants and cultivated as thick pellicle growth on the Doub and Youmans liquid medium (499). (Appendix I.a).

Salmonella enteritidis strain was used to study specificity of immunization with *M.vaccacae* -(0:1,9,12 H:g,m)The strain was obtained from National collection of type culture (NCTC), Colandale Avenue, London.

A tube containing lyophilised culture was transferred to 0.5ml. peptone water under sterile conditions. The suspension was transferred to 4 ml. Peptone water and was incubated at 37°C for 3h. The grown culture was then transferred in to liquid broth (Appendix I.b) and incubated at 37°C for 18h on shaker.

Preparation of Mycobacterial cell suspension for immunization.

The flasks containing surface growth of *M vaccae* after 10 days of incubation at 37° C , were swirled so as to disturb the pellicle and the cell mass was allowed to settle at the bottom. Contents were pooled in to one flask and supernatant discarded . The cells were inactivated by Gamma radiation in a Co⁶⁰ radiation chamber with a total dose of 2.4 Megarads. The irradiated cells were then washed with sterile phosphate buffered saline (PBS) pH 7.2 (Appendix I.c) and centrifuged at 12,000g for 30 min. One ml of pelleted cells (by centrifugation) were finally suspended in 50 ml sterile PBS to give a suspension of 2×10^9 bacilli/ml . A vaccine containing 10^8 cells in 0.05 ml PBS was prepared for intradermal (i.d.) immunization and 0.5 ml for intraperitoneal (i.p.) immunization. The i.d. immunization was given in flanks.

Preparation of Sonic Extract

As test antigen for *in vivo* and *in vitro* assay.

10 day old culture of *M vaccae* was harvested and subjected to 2.4 Mrad gamma radiation in a Co ⁶⁰ radiation source , these were then washed in PBS and disrupted by sonication (Branson Sonifier, B30) with a 50% duty cycle at 20kHz for 45 min. , keeping the tube constantly on ice. The sonicate was then centrifuged at 50,000g (Sorval OTD Combi - Ultracentrifuge) for 1 h to remove cell debris. Cell free supernatant was filtered through 0.22 µm membrane filter (Millipore, USA) and its protein content was estimated by Lowrys method (500).(Appendix II.a). After confirming the sterility the

sonicate was aliquoted and stored at -40°C and used as test antigen for DTH and for lymphoproliferative assay.

The salmonella culture was also subjected to γ radiation, washed in sterile PBS and sonicated for 45min. at 20kHz in the same manner. The sonicate was filtered, aliquoted and was used to test for DTH, lymphoproliferative assays to confirm the specificity of immunization with *M.vaccae*.

Preparation of Lysozyme-Papain digested sonicate for preparation of 65kDa antigen.

Gamma irradiated cells of *M vaccae* were washed in 10 mM Tris HCl buffer pH 7.8 (Appendix I.d). The sonicate was centrifuged at 750g (Centricon T34) for 10 min to remove unbroken cells. The supernate was collected and was treated with Lysozyme (SIGMA) (2mg/ml concentration) (501) and incubated at 37°C water bath for 2 h. 10 mM EDTA was added to the mixture for the enzyme reaction. [A small aliquote of the sonicate was removed and spun at 50,000g for 1 h and its protein content estimated]. The Lysozyme treated sonicate was subjected to Papain digestion by adding 1 mg Papain (SIGMA) for 15 mg of protein content in the sonicate. 0.1M L-Cystein HCl (0.5ml/30ml of mixture) was added to facilitate the digestion. The digestion was allowed to proceed at 37°C for 2 h in the water bath with intermittent shaking. The reaction was then stopped by transfer of the flask to a chilled ice bath and addition of 0.13M iodoacetamide in the proportion of 0.2ml for every 10ml of reaction mixture. The suspension was centrifuged at 50,000g for 1h at 4°C , supernate collected, its protein content estimated and was then aliquoted and stored at -20°C .

Purification of 65-kDa antigen of *M vacccae*

The 65kDa antigen of *M.vacccae* was purified by preparative isoelectrofocussing (IEF) :

This step was undertaken , based on earlier described pI of the 65-kDa antigen to be 4.3 (502). Preparative IEF was done on 1% gels of agarose (SIGMA IEF grade) employing 2% Ampholine (pH range 3.5-5.0) by the method of Vesterberg (503) . 20ml distilled water containing 1 % Agarose (IEF grade) with sucrose (12.5% w/v) , was boiled to dissolve the agarose, to which 2 % Ampholine of pH 3.5-5 was added after cooling . The slurry was layered on a gel bond film for agarose (SIGMA) of the size 7.5 cm X 7.5 cm and the gel allowed to solidify . Filter paper strips (Whatmwn No 17) dipped in appropriate electrolytes were placed on either end of the gel. Anodal electrolyte was 0.05M H₂SO₄ pH 1.5 and the cathodal electrolyte - 0.4M HEPES, pH 5.2. Prefocussing was carried out at 12mA current and 250 voltage, for 30min. A trough of size 0.5 cm X 6.0 cm was cut at the cathodal end and approximately 1 ml of lysozyme -papain digested sonicate containing 15mg/ml protein was added in it. Electrofocusing was carried out at 3 Watt constant power for 3h, after which current was allowed to fall to gradually to zero. The entire electrofocussing was carried out at 4⁰ C which corresponds to 65-kDa antigen, was cut , crushed in to fine particle and PBS Tween 80 was added to it and the protein was allowed to elute overnight at 4⁰ C . Eluted protein from several gels was pooled, the molecular weight of 65 kDa confirmed by SDS -PAGE. (Appendix II.b).

Preparation of 65-kDa antigen bearing nitrocellulose membrane (NCM) particles

Electroblotting of the 4.3 pI band from the IEF gel.

The Lysozyme-papain digested sonicate was subjected to preparative agarose IEF. The focussed agarose gel was then blotted on to a nitrocellulose membrane (NCM, SIGMA) employing Midget multiblot Electrophoresis transfer unit (LKB, Pharmacia Sweden) as described below.

Nitrocellulose membrane, 7.5 cm. X 7.5 cm. in size was cut and soaked in Tris-glycine pH 8.4 Transfer buffer (Appendix I.e). Sheets of thick (Whatman No. 17) and thin (Whatman No 1) filter paper of the size 9 cm. X 9 cm. were cut and also soaked in transfer buffer. The focussed agarose gel was soaked in transfer buffer for 30 min. at 4° C for equilibration.

In the transferring Cassettes provided with the blotting equipment, following assembly was arranged. The agarose gel was gently placed on a double layer of thin and thick papers, with the gel bond film facing upwards. The latter was then gently stripped off . The exposed surface of the gel was then covered with NCM . This was then covered with a thin and thick filter paper . The entire assembly was then covered on both sides with sponges (presoaked in transfer buffer) The assembly was loaded onto a cassette , which was then loaded in to a transfer unit such that NCM was on the anodal side of the gel. The chamber was filled with transfer buffer and the whole unit was kept on magnetic stirrer for uniform mixing of the buffer. The electrophoresis was carried out at 80mA constant current for 2 h at 4° C. After the run NCM was gently removed and a

strip of 1cm width was cut and stained with India ink to mark the position of 4.3 pI band.

Preparation of Suspension of NCM adsorbed band.

The preparation of suspension of NCM particles bearing bound protein, to support lymphoproliferation , was done by the method described by Abou Zeid *et al* (504). The NCM strip corresponding to the 4.3 pI band was cut in to fine small pieces and transferred to a test tube . Dimethyl Sulfoxide (DMSO, SIGMA) was added in the proportion of 250 μ l/20mm² area of NCM and the mixture kept for 1h at room temperature to dissolve the NCM strip completely. The dissolved NCM with the adsorbed antigen was precipitated out as small particles by adding equal volume of Carbonate buffer, 0.05M pH 9.6 (Appendix I.f) . The buffer was added dropwise slowly with continuous mixing on the vortex mixer . The suspension was then centrifuged at 10,000g for 10min at 4^o C and the pellet washed with RPMI 1640, resuspended in a minimum volume of RPMI 1640. This preparation was titrated for its ability to support lymphoproliferation of spleen cells of mice primed i.d. with *M vacccae* , 100 μ l of the optimum dilution of the suspension was employed in all the assays.

Delayed type hypersensitivity testing (DTH)

DTH test was done by the method of Gray and Jennings (505). A 0.03 ml volume of the *M.vacccae* sonicate containing 2mg/ml protein was injected in to the hind foot pad of normal and immunized mice . Measurement of foot pad thickness was taken before (0 h) , 24h and 48h after the injection. The results were expressed as corrected foot pad enlargement (CPE) , which is the difference between the foot pad enlargements caused by the test

antigen in the immunized and control mice simultaneously tested. Results were analyzed by students "t" test, by comparing the CPE values with zero.

Lymphoproliferation test

This assay was carried out employing standard microculture technique (506). Normal or immunized mice were sacrificed by cervical dislocation, their spleens removed and teased and a single cell suspension was prepared in RPMI 1640. The cell suspension was centrifuged at 400g for 10min to pellet the cells. The pellet was suspended in RPMI 1640 and layered on Ficoll-hypaque (Density 1.083 ± 0.001 SIGMA) and centrifuged at 400g for 20min at room temperature. The mononuclear cells at the junction were separated and washed thrice with RPMI 1640. Viability of cell suspension was assayed by trypan blue exclusion test. Viable cells were suspended at 2×10^6 cells/ml in the culture medium RPMI 1640, containing 5% FCS, 2.0mM L-Glutamine (SIGMA) and 0.5% Antibiotic Antimycotic (SIGMA, which in the final concentration of RPMI gives 50 units Penicillin, 50 μ g Streptomycin, 0.012 μ g Amphotericin B per ml). Each well of the tissue culture plate (Nunc, Denmark 96 U) received 2×10^5 cells and requisite amount of antigen; *M vaccae* sonicate, 65kDa antigen bearing NCM particles; or mitogens Concanavalin A (Con A SIGMA 2 - 20 μ g/ml) or superantigen; Staphylococcal enterotoxin B (SEB, SIGMA 1-10 μ g/ml) in the final volume of 200 μ l/well. Control wells did not receive any stimulant. The concentration of stimulants added varied in each experiment and are given in detail along with experimental results. Cultures were incubated in a CO₂ incubator (Flowlab) at 37° C and 5% CO₂ in humidified air. 1 μ Ci of ³H labelled Thymidine (BARC, India specific activity 6.5 Ci/mMole) was added 48-72h later for Con A

and SEB and 120h later for antigen. After 18h of pulsing the cultures were harvested on Whatman 3mm filter paper discs (20mm diameter), air dried and soaked in 10% trichloroacetic acid for 10min. These were further washed in distilled water thrice and finally in 20% methanol to wash off unincorporated isotope . Filter discs (which retained predominately macromolecules with incorporated isotope) were dried and were transferred in to vials containing Scintillation fluid (Appendix I.g). Incorporation of the label assayed in a β - Scintillation counter (Rackbeta 1211, LKB) and expressed as counts per minute (CPM) . Cultures were run in triplicates.

Separation of T cells

T cells were separated using detoxified, preactivated nylon wool columns by the method described by Julius *et al* (507). 0.6gm of dry Nylon wool (Leukopak, Fenwel lab , USA) was cut and kept under running tap water overnight. Washed wool was than transferred to distilled water and given frequent washes for 2 days. The wool was teased and soaked in RPMI 1640 for 7 - 8 h at 37° C with three changes. RPMI treated wool was thoroughly rinsed with distilled water for 1 day at 4° C . This detoxified wool was further boiled and packed in 10 ml. syringes each. The syringe with the wool was sterilized by autoclaving.

At the time of the assay , the sterilized syringe with detoxified nylon wool was kept vertical at 37° C and washed with ten volumes of RPMI 1640 medium. 0.5 - 0.8 ml. suspension of 10^8 lymphoid cells were gently layered on the column and incubated for 30 min. at 37° C . The non adherent cells were eluted with RPMI at 6 - 10 drops/minute. The eluted cells were then chilled , washed in RPMI

and viability estimated by trypan blue exclusion . These were used as T cells in the lymphoproliferation assays.

Irradiation of spleen cells for use as antigen presenting cells (APC's)

Lymphocytes separated from immunized or normal mice were subjected to 800 rad gamma radiation in a Co⁶⁰ source. The irradiated cells were washed in RPMI 1640 and used as APC's.

Monoclonal antibodies

Murine hybridoma culture fluids of anti - mouse TCR V β 14 were kindly supplied by Dr. S. Rath, National Institute of Immunology, Delhi. 25 - 50 μ l of neat supernatants of hybridoma cultures were added to the test lymphoid cultures.

Murine hybridomas of anti-mouse specificities, Ia^k, IE (all Alleles), anti-thy 1.2, anti-L3T4 and anti-Lyt2.2 were kindly supplied by Dr. K.B.Sainis, Immunology, BARC, Mumbai.

Preparation of IL-2 containing culture supernatant.

Normal rat spleen cells were stimulated with Con A and culture supernatant containing IL-2 (Con A Sup) was prepared and IL-2 content assayed by the procedure described earlier (46) (Appendix II.c). 25 μ l of the medium containing 270 - 540 IU /ml. IL-2 were added to the culture wells for the assay of IL-2 induced spleen cell proliferation.

Antibody against 65-kDa antigen of *M vaccae*

Raising antiserum against 65-kDa antigen in Rabbit

The lysozyme-papain digested *M.vaccae* sonicate was subjected to preparative agarose IEF as described earlier and the visible precipitin band at pI 4.3 was cut. The bands from three such gels were pooled in a glass tube and homogenized to make a suspension. To this equal volume of Freund's incomplete adjuvant (SIGMA) was added and a homogenous emulsion prepared by thoroughly mixing for 30 - 45 min. A rabbit weighing 2 kg was depilated and the antigen preparation was injected intradermally at multiple sites on the back. The immunization was repeated twice at 10 days interval. 7 days after the last immunization the rabbit was test bled from ear artery. Serum separated was tested against *M.vaccae* sonicate and 65-kDa antigen by agar gel diffusion method of Ouchterlony (508). After getting a high titre, the rabbit was bled out, serum separated and decomplexed 56°C for 1h. IgG fraction was precipitated by 33% saturation with ammonium sulphate. The precipitate was centrifuged and dialysed against carbonate buffer (coating buffer- Appendix I.f) for removal of sulphate ions. The protein content was estimated by absorbance readings at 280 nm and aliquoted and stored at -20°C.

Assay for anti 65-kDa antibody levels

Antibody levels against 65-kDa antigen of *M.vaccae* in the sera of immunized mice were assayed by indirect sandwich ELISA technique.

ELISA system - ELISA plate wells (Nunc, Denmark) were coated with rabbit antibody against 65-kDa antigen to capture the

65-kDa antigen from *M. vaccae* sonicate. Test mouse serum samples (and positive and negative controls) were added on to the complex. The anti 65-kDa antibodies in test mouse sera were detected by using an anti- mouse IgG specific peroxidase conjugate.

Reagents -

1. Coating antibody - IgG fraction of rabbit antiserum against 65kDa antigen of *M.vaccae* in coating buffer.
2. Lysozyme papain digested sonicate of *M.vaccae* as a source of 65 kDa antigen
3. Positive control serum - Serum of mice immunized i.d. with 65 kDa antigen of *M.vaccae* in Freund's incomplete adjuvant given three times at one week interval.
4. Anti-65 kDa antibody containing known standard mouse antiserum. Mice given thrice i.p. immunizations with 10^8 *M.vaccae* cells generated a good anti-65 kDa antibody response . A pooled serum of several i.p. immunized mice was used for the standardization of the assay . This serum was employed as a standard known antiserum. It was stored at -40°C in small aliquots. Everytime an assay was performed a fresh aliquote was employed.
5. Normal mouse serum.
6. Anti - mouse IgG specific HRP conjugate (SIGMA) - Affinity isolated goat anti-mouse IgG (heavy chain specific) antibody coupled to horse radish peroxidase.
7. Substrate solution- OPD (o-phenylenediamine dihydro-chloride) 4mg /10ml. Sodium citrate buffer , 0.15M pH 6.0 (Appendix I.h) $2\mu\text{l}$ of H_2O_2 added and substrate solution used immediately.
8. Dilution buffer - PBS T-20 (Appendix I.i).
9. Washing solution - Saline T-20 (Appendix I.j).

Procedure - Wells of ELISA plates (C12 Maxisorp- Nunc , Denmark) were coated with 100 μ l of IgG fraction of rabbit antiserum against 65kDa antigen. The plates were incubated overnight at 4^o C and for ½ h at 37^oC next day. The well contents were discarded and well surfaces were blocked with 2% gelatin in coating buffer for 1 - ½ h at 37^o C. The plates were washed 5 - 6 times with washing solution, saline T -20 on an ELISA washer (Organon Teknika, miniwasher) and 100 μ l of Lysozyme-papain digested *M.vacccae* sonicate was added to the wells and incubated at 37^oC for 2h. The wells were then washed 5-6 times and 100 μ l of different dilutions of test mouse serum samples along with appropriate positive and negative controls were added to the wells. Positive control and standard antibody containing mouse sera were used at 1:8 dilution. PBS T-20 was used as negative control and normal mouse serum at 1:8 dilution were also added as a separate set of negative control. The plate was incubated at 37^oC for 2h . The plate was washed 5-6 times and 100 μ l of anti mouse IgG HRP conjugate (1:10,000 dilution) was added to the wells and incubated at room temperature in the dark for 10-15 min. After the colour development , the reaction was stopped with 2 M H₂SO₄ and read at 492 nm in a ELISA plate reader.

$$\text{Antibody titre} = \frac{A_{492} \text{ of test serum sample}}{A_{492} \text{ of standard mouse serum}} \times \text{dilution of test serum} \times 10$$

Abs units/ml.

The antibody titre was expressed as absorbance units/ml (AU /ml)

As there was variation in absorbance value at each test , the absorbance was expressed as a multiple of a single source standard mouse serum with reasonable amount of anti 65-kDa antibody. Thus

the results of antibody titres of serum samples done on different occasions were normalized with respect to the same standard.

Lymphokine Assay

Lymphokines IL-2 , IL-4 and IL-10 in culture supernates were assayed by ELISA employing commercial ELISA kits of Genzyme and Pharmingen.

IL-4and IL-10 ELISA - These assays were performed employing kits purchased from Ms Pharmingen , USA .

ELISA system - Plates were coated with purified rat anti-mouse IL-4/IL-10 monoclonal antibody (Ab) to serve as capture antibody. On to this recombinant murine IL4/IL10 as standards or samples were added. The lymphokines were detected by adding biotinylated rat anti-mouse

IL-4/IL10 antibody followed by Streptavidin horse raddish peroxidase HRP conjugate and o-phenylenediamine (OPD) substrate there after.

Reagents

1. Capture antibody

- a) Purified monoclonal rat anti IL-4 antibody (isotype IgG₂b) (Clone -BVD4 - I D 11)
- b) Purified monoclonal rat anti IL-10 antibody (isotype IgG₁) (Clone JES 5 -2A5)

10 µl of anti-IL-4 Ab and 20 µl of anti-IL-10 Ab solution were diluted to 2 ml. With 0.1M NaHCO₃ pH 9.6, the coating buffer to get 5 µg/ml concentration of antibody protein.

2. Lyophilised recombinant murine IL-4 and IL-10

The lyophilised powder was first reconstituted with sterile distilled water and then diluted with PBS T-20 containing 3% BSA, aliquoted and stored at -20° C. One aliquote was further diluted with conjugate stabilizer (Pierce, USA) to give 50ng/ml concentration of each cytokine, which was used as standard stock of recombinant IL-4/IL-10. These were aliquoted and stored at -20° C and were used at 0.2 - 1 ng range at the time of the assay as known standards.

3. Secondary antibody

- a) Biotinylated rat anti-mouse IL-4 monoclonal antibody (Clone BVD624G2) (isotype IgG₁)
- b) Biotinylated rat anti-mouse IL-10 monoclonal antibody (Clone SxC 1 (isotype IgM)

10 μ l containing 10 μ g of antibody was diluted to 5 μ g/ml.
With PBS T-20 containing 3% BSA.

4. Streptavidin horse radish peroxidase (HRP) conjugate -

2 mg of protein was reconstituted with 2 ml. of PBS T-20 which was further diluted to 10ml. With conjugate stabilizer (Pierce, USA) to get final concentration of 200 μ g/ml. , aliquoted and stored at -20^o C. The aliquote was diluted 1000 times at the time of the assay to give 0.2 μ g/ml.

5. Conjugate Stabilizer solution (Pierce ,USA) -

was employed for dilution of conjugates and the cytokine standards for storage.

6. Superblock blocking buffer in PBS (Pierce , USA) -

was employed for blocking unbound sites on the surface of ELISA plate wells.

7. Substrate Solution -

4 mg of o-phenylenediamine dihydrochloride (OPD) was dissolved in 10ml. Citrate buffer, to which 2 μ l of H₂O₂ was added.

Procedure - 50 μ l of Capture antibody (5 μ g/ml.) was delivered in each well of the ELISA plate (Maxisorp Nunc,Denmark) and incubated overnight at 4^o C, and at 37^o C for ½ h next day. The plate was washed twice with PBS T-20 (Organon Teknika miniwasher). Superblock blocking buffer was added to bind the uncoated surfaces for 1h at room temperature. The plate was washed twice again with PBS T-20. 100 μ l of culture supernates, for assay of

cytokine content were added to the wells. 100 µl of dilutions of standard recombinant IL-4/IL-10 were added to the respective wells as known standards and incubated at room temperature (RT) for 4h. After the incubation the plate was washed 4 times and 100 µl of biotinylated anti-mouse IL-4/IL-10 were added to each well and incubated at RT for 45 min. The plate was washed 6 times with PBS T-20 and 100 µl of Streptavidin HRP conjugate was added to all the wells and incubated for 30 min. at RT. The plate was washed 8 times with PBS T-20 . 100 µl of substrate solution was added to each well and incubated in the dark for 15 min. at RT for colour development. The reaction was stopped with 2.5 M Hcl and readings were taken at 492 nm in an ELISA reader (Titertek Multiscan - Flowlab).

Calculations - Cut of value was arrived at by the formula
Mean absorbance of negative control + 3S.D. (Standard deviations)
Amount of Lymphokine was calculated by the following formula

$$\text{Lymphokine IL-4/IL-10 (pg)/10}^6\text{cells} =$$

$$\frac{A_{492} \text{ of sample}}{A_{492} \text{ of standard}} \times \frac{\text{concentration of lymphokine standard (pg/ml)}}{\text{volume of culture well (ml)}}$$

The lymphokine levels were expressed as pg/10⁶ cells cultured.

IL-2 ELISA - This assay was performed employing kits purchased from Ms Genzyme, USA.

Design of the assay - Microtitre plates precoated with anti-mouse IL-2 monoclonal antibody were provided. Different dilutions of standard recombinant mouse IL-2 or culture supernates were

added to them. The IL-2 levels were detected by addition of biotinylated polyclonal anti-mouse IL-2 followed by Streptavidin peroxidase conjugate.

Procedure - The assay was carried out as per the instructions given in the Instruction Booklet provided along with the kit.

Detection of anti-ds DNA antibodies indirect ELISA

Antibodies against dsDNA were detected by indirect ELISA as described by Macanovic (509). The ELISA plates were coated by overnight incubation at 4°C with calf thymus DNA (Sigma), 5µg/ml in 1mM EDTA/PBS (Appendix I.k). (100µl/well). The wells were blocked with 3% BSA for 30min at room temperature. The plates were washed with wash buffer -1mM EDTA/PBS containing 0.05% Tween 20. 100µl of test sera, normal (B6 x C3H)F1 sera, and positive control serum, pre diluted 1 in 200 with the wash buffer, were delivered to each well in triplicate and incubated at room temperature for 60min, after which they were washed. Washed wells , then received HRP conjugate of goat anti-mouse IgG (Sigma- 1:1000 dilution), and incubated at RT for 60min after which substrate OPD-H₂O₂ were added to the well, incubated for 30min for colour development. The reaction was stopped with 2N H₂SO₄ and read at 450 nm. As positive control, pooled serum of 7 months old NZB/WF1 mice was employed.

Results were expressed as ratio of A₄₅₀ of each test serum to that of NZB/WF1 pool. Samples with ratio of 0.25 or greater were considered to be positive for anti-ds DNA antibodies.

RESULTS

This study is in two parts

PART I : Study of mechanism of CD4 + T helper (Th) cell non responsiveness to foreign antigens in chronic GVHD

The chronic GVHD is associated with characteristic immunological abnormalities namely,

1. Inability to mount a self MHC restricted CD4 + Th response to foreign antigens even though the response to allogeneic MHC or polyclonal T cell mitogen is unaffected (57).
2. Despite the above immune defect, the individuals / experimental animals afflicted with this disease reveal evidence of chronic activation of immune system, as manifested by generation of autoantibodies to red cells, DNA and many other self antigens and lymphoid hyperplasia.

In the light of these findings it is logical to suspect that the above immune defect could be due to activation induced cell death (AICD) of CD4 + Th cells. Hence the chronic GVHD experimental model was employed to study mechanism of CD4 + T cell non responsiveness to foreign antigens.

PART II : Study of role of superantigen derived from infectious mouse mammary tumor virus (MMTV) in activation of CD8 + T suppressor (Ts) cells by an unrelated bacterial antigen

Our earlier studies on emergence of antigen specific non responsiveness to immunization with a vaccine of *M. vaccae* by the intraperitoneal (i.p.) route, revealed that the non responsiveness was due to expansion of antigens specific CD8 + Ts cells (4,41). It was further shown that the CD8 + Ts cells induced suppression by competition with simultaneously primed CD4 + Th cells for IL - 2(46). For reasons discussed later, when the experimental model will be described in details, a role for exogenous or endogenous superantigens in activation of CD8 + Ts cells by the bacterial antigens was suspected. The second part of the thesis deals with this issue.

Both these phenomena deal with activation induced non responsiveness to an antigen.

PART I

Study of mechanism of CD4 + T helper (Th) cell non responsiveness to foreign antigens in chronic GVHD

I.1 Establishment of chronic GVHD in mice

GVHD develops when immuno competent lymphocytes are injected into an allogeneic recipient whose immune system is compromised or by parental spleen cells into healthy adult unirradiated F1. For the successful induction of chronic GVHD, it is crucial to avoid the severe hemopoietic depletion of acute GVH reaction. A convenient way to achieve chronic GVHD is to remove cytotoxic CD8 + T cells from the donor cells or to use cells of DBA/2 strain for induction as the number of CD8 + T cells capable of causing acute GVHD is low (474,480). In our experimental model, (B6 female X C3H male) F1 mice have been utilized. For induction of chronic GVHD, it was decided to employ C3H parental spleen cells. Since C3H and B6 differ at both MHC class I and class II loci, transfer of C3H spleen cells would invariably induce acute GVHD and not chronic GVHD (476). It was therefore necessary to titrate the dose of C3H parental spleen cells, at which number of CD4+ T cells transferred would be optimum for induction of chronic GVHD and number of CD8+ T cells would be too low to induce acute GVHD.

a) Induction of chronic GVHD.

Normal parental C3H mice were sacrificed, spleens harvested and single cell suspension made. Different numbers of normal spleen cells 5×10^6 , 10^7 , 2×10^7 , 5×10^7 , 10^8 , 5×10^8 and 10^9 were transferred into 8 - 10 week old (B6 X C3H)F1 by tail vein injection and the mice were observed for a period up to 6 months.

b) Assessment of chronic GVHD.

Establishment of chronic GVHD was confirmed by two types of parameters

- 1) GVH index as described by Simonsen (510). This is a simple biometrical consideration to the spleen weight (511), which is based on the finding that the degree of splenomegaly due to lymphoid proliferation, which develops in the early stages of GVH reaction is correlated with the number of immunologically competent donor cells and generation of chronic GVHD (57) and
- 2) Generation of autoantibodies against autologous red cells (Appendix II.f) and double stranded (ds) DNA .

Normal animals and those under test were given liberal feed and water for 24 hrs, were weighed and then sacrificed. Their spleens were harvested and weighed. The spleen weight was expressed as a fraction of body weight. The spleen weight to body weight ratio of each experimental animal was compared with the mean spleen weight to body weight ratio of normal mice and expressed as GVH index.

$$\text{GVH index} = \frac{(\text{Spleen weight / body weight}) \text{ of test mouse}}{\text{Mean (Spleen wt/body wt) normal F1}}$$

The above ratio, when greater than 1.34 was considered as an index of chronic GVHD.

Six (B6 x C3H)F1 mice with chronic GVHD having GVH indices greater than two were selected, and were bled from the retro orbital plexus. Serum was separated and tested for auto

Table I.1

Induction of chronic GVHD in (B6 x C3H)F1 mice

Expt No.	No. of mice tested	No. of mice showing chronic GVHD	GVH indices	Autoantibodies*	
				red cells	ds DNA
1.	10	10	1.6, 1.7, 2.0, 1.5, 1.7, 1.8, 1.5, 1.5, 1.9, 2.8	ND	ND
2.	10	8	4.4, 2.2, 3.2, 2.0, 2.6, 2.8, 1.8, 2.2	ND	ND
3.	10	8	1.6, 3.0, 2.1, 2.2, 2.5, 2.5, 4.4, 3.2	ND	ND
4.	6	5	2.5, 2.9, 2.0, 2.6, 3.2	5/6	5/6

Normal C3H mice were sacrificed, spleens harvested and single cell suspension made. 5×10^7 cells were injected by tail vein into 8-10 week old normal (B6 x C3H)F1 mice. Three weeks later the mice were weighed, sacrificed, spleens harvested and weighed and GVH indices calculated.

* No. reactive /No. tested . All the six mice tested, had received the parental C3H cells three months earlier.

antibodies against red cells by indirect Coomb's test. The sera were also tested for antibodies against ds-DNA by indirect ELISA.

c) Results of the experiments

The GVH indices in 4 different experiments are shown in (Table I.1). Small number of lymphocytes i.e. 5×10^6 , 10^7 , 2×10^7 showed no effect, whereas higher doses, 10^8 , 10^9 cells induced acute GVHD with lymphoid atrophy and death. 5×10^7 cells were found to be optimum for the induction of chronic GVHD consistently in the recipients. Everytime chronic GVHD was induced, the GVH index was calculated to ensure that the mice were indeed suffering from chronic GVHD.

Animals with GVH indices greater than 1.34, typically generated autoantibodies against red cells as indicated by strongly positive indirect Coomb's test showing the titre of 1:64 or greater. Animals also showed antibodies against ds DNA.

I.2 T-Helper responses to *M. vaccae* are lost in mice having chronic GVHD of 3 months duration but are normal in mice having the disease for only 3 weeks

Normal C3H were sacrificed and 5×10^7 spleen cells were injected i.v. into the 8-10 week old (B6 X C3H)F1 mice to induce chronic GVHD. Normal (B6 X C3H)F1 mice and those having chronic GVHD of 3 weeks and 3 months duration (5 per group) were immunized i.d. with a vaccine containing 10^8 *M.vaccae*. One week later their response to the sonicate of the organism tested. Mice were then sacrificed, spleens removed and weighed to calculate GVH index. Single cell suspensions made and cultured with sonicate of *M.vaccae* and a standard lymphoproliferative assay performed.

Proliferative response of spleen cells to the polyclonal mitogen Concanavalin A (Con A) was also simultaneously tested.

Normal (B6 X C3H) F1 mice mounted a DTH response to *M.vaccæ* and the mice with chronic GVHD of 3 months duration did not. DTH response of mice with chronic GVHD of only 3 weeks duration was comparable to that of normal mice (Table I.2).

The *in vitro* antigen specific lymphoproliferative responses were similar to the DTH responses. The spleen cells of normal i.d. primed (B6 X C3H) F1 mice showed an excellent *in vitro* proliferative response on stimulation with antigen. The spleen cells of i.d. primed F1 mice having chronic GVHD of 3 weeks duration also showed a normal *in vitro* proliferative response. In contrast, the response of i.d. primed F1 mice having chronic GVHD of 3 months duration was poor. However, the stimulation indices of the cells of mice with chronic GVHD were very low because of high level spontaneous background proliferation without any antigenic stimulation. This is expected because of reaction induced by the parental T and B cell clones recognizing the alloantigens of the other parent on F1 cells. In order to resolve this difficulty, the i.d. primed spleen cells of mice with chronic GVHD were passed over a nylon wool column to obtain i.d. primed T cells of mice with chronic GVHD. These were mixed with equal number of syngeneic irradiated naive spleen cells of normal mice to serve as APCs and cultured with *M.vaccæ* antigen and a proliferative assay performed. This modification in the assay procedure reduced the level of background proliferation considerably. The T cells of i.d. primed mice with chronic GVHD of only 3 weeks duration, which showed a practically normal proliferative response to restimulation *in vitro* with the antigen, also showed a high stimulation index, comparable

Table I.2

DTH and antigen specific and polyclonal proliferative responses of mice with chronic GVHD.

Mice	DTH CPE (mm) ± SE	Proliferative response CPM ± SE (SI)		
		<i>M.vaccæ</i> sonicate	Con A	
		2 × 10 ⁵ whole spleen cells	10 ⁵ T * cells	2 × 10 ⁵ whole spleen cells
Normal (B6XC3H)F1	0.29 ± 0.01	12,060 ± 2,129 (14.4)	18,760 ± 1,437 (17.1)	1,13,308 ± 3,043 (60.2)
Chronic GVHD 3months	0.08 ± 0.02	2,888 ± 675 (2.4)	2,980 ± 248 (4.5)	43,264 ± 5,507 (7.9)
Chronic GVHD 3 weeks	0.27 ± 0.04	7,019 ± 793 (2.1)	9,668 ± 1,107 (11.7)	38,029 ± 840 (8.2)

(B6 × C3H)F1 mice, Normal and those with chronic GVHD of 3 weeks and 3 months duration , were immunized i.d. with killed *M.vaccæ*. One week later , the DTH responses of the mice to the sonicate of *M.vaccæ*, were tested. The mice were then sacrificed, spleens harvested and single cell suspension made. These were cultured with sonicate (20 µg/ml) for 5 days or Con A (5µg/ml) for 48h, pulsed with ³H labelled thymidine for 16h and incorporation of label assayed.

**M.vaccæ* primed spleen cells were also passed over nylon wool column to obtain i.d. primed T cells which were mixed with equal number of irradiated spleen cells of normal F1 mice and cultured with *M.vaccæ* sonicate and a proliferative assay performed as before.

SE - Standard error

SI - Stimulation index = mean CPM with Ag/ mean CPM without Ag.

CPE - Corrected foot pad enlargement. CPM - Counts per minute.

to that of primed cells of normal mice. In contrast, the stimulation index of the primed spleen cells of mice with chronic GVHD of 3 months duration did not improve by this modification.

The *in vitro* proliferative response to the polyclonal mitogen Con A was also depressed in chronic GVHD, however, the depression was marginal. Chronic GVHD of 3 months and 3 weeks duration responded in a similar fashion to Con A. As seen with antigen specific proliferative response, the stimulation indices of Con A induced proliferation, despite reasonable proliferation, were also low, because of high background proliferation without any mitogen (Table I.2).

This experiment indicated that self MHC restricted T-h cell responses are lost in chronic GVHD of 3 months duration as reported earlier (57). However, it is significant to note that such a T cell response to antigens is preserved in animals having chronic GVHD of 3 weeks duration only. The response to polyclonal mitogen was not affected to the same extent in mice harboring chronic GVHD of either 3 weeks or 3 months duration.

I.3 Effect of repeated immunization on T helper cell responsiveness to *M.vaccae* of mice harboring chronic GVHD of 3 weeks

a) Cell mediated responses

Chronic GVHD was induced in (B6 X C3H)F1 mice as before and after 3 weeks these mice, along with normal (B6 X C3H) F1 mice, were immunized with 10^8 killed *M.vaccae* by the i.d. route. The immunization was repeated twice more at 7 days interval, DTH and antigen specific proliferative responses of the mice were tested

one week after each immunization. Lymphoproliferative assay was done with whole spleen cells and splenic T cells with irradiated normal spleen cells as APCs. As antigen, both sonicate and the immunodominant 65-kDa antigen of *M.vaccae* were employed as stimulating antigen.

Repeated immunization of normal F1 mice with *M.vaccae* showed an increase in the DTH response to the sonicate. In contrast, the DTH responses of mice with chronic GVHD of only 3 weeks duration, progressively declined with each immunization, being practically negative after the third immunization (Fig 1).

The proliferative responses against *M.vaccae* sonicate also showed similar pattern. Normal (B6 X C3H) F1 mice showed excellent proliferative response with repeated immunization whereas mice with chronic GVHD of 3 weeks duration showed a progressive decline with repeated immunization (Fig 2). Tests of proliferative response with 65-kDa antigen gave similar results.

b) Specificity of loss of T cell responsiveness

In order to see whether the observed loss of Th cell responsiveness against *M.vaccae* was specific towards the organism, we checked the immune response of these animals to another immunogen namely *S.enteritidis*.

Both normal F1 and those with chronic GVHD of 3 weeks duration were given two i.d. immunizations with *M.vaccae* at one week interval. One week after the second immunization mice were divided into two groups. One group of normal F1 and chronic GVHD mice received third immunization with *M.vaccae* and the other group of normal F1 and chronic GVHD were given i.d.

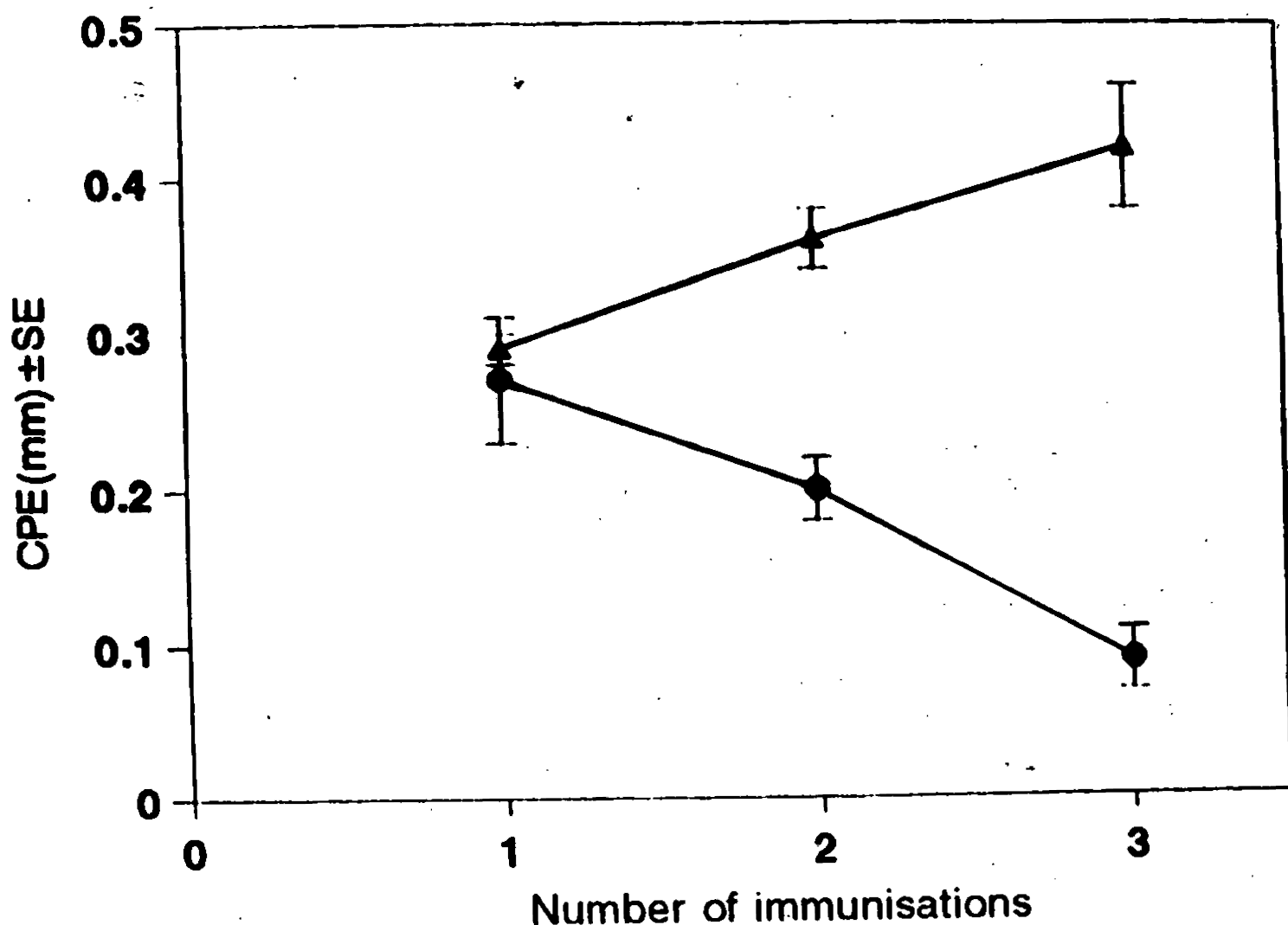


Fig 1 DTH response of mice on repeated immunization with *M.vaccae*.

(B6 x C3H)F1 mice, normal [▲] and those with chronic GVHD of three weeks duration [●] were given three successive immunizations with 10^8 *M.vaccae* by the i.d. route at one week intervals. One week after each immunization the DTH response of mice to *M.vaccae* sonicate was tested.

CPE - corrected foot pad enlargement
SE - standard error.

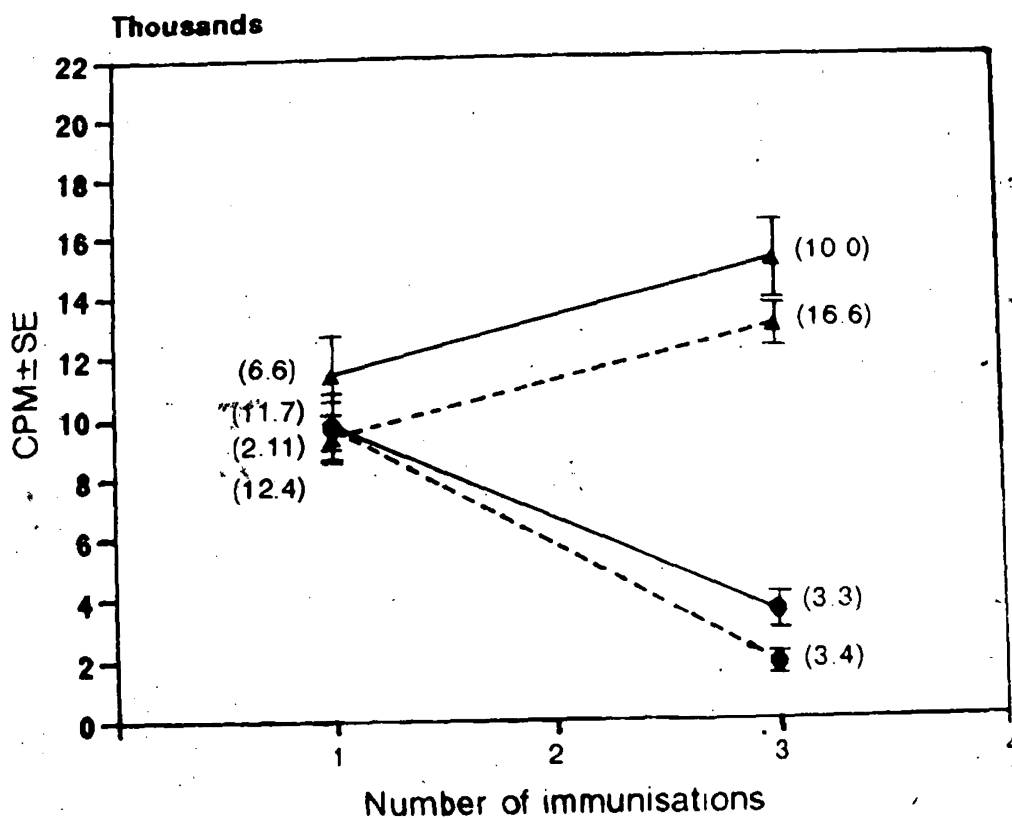


Fig 2 Lymphoproliferative responses of mice on repeated immunization with *M.vaccae*

(B6 x C3H)F1 mice, normal and those with chronic GVHD of three weeks duration were given three successive immunizations with 10^8 *M.vaccae* by the i.d. route at one week intervals. One set of mice, of each type, were sacrificed 7 days after the first immunization and the remaining sacrificed 7 days after the third immunization. The spleens were harvested and a lymphoproliferative assay performed against 65-kDa antigen of *M.vaccae*. For the assay either 2×10^5 whole spleen cells or 10^5 T cells of the primed mice were employed. Irradiated normal F1 spleen cells were employed as APCs in the cultures with primed T cells.

[—▲—▲—▲—] Response of primed whole spleen cells of normal mice.

[---▲---▲---▲---] Response of primed splenic T cells of normal mice.

[—●—●—●—] Response of primed whole spleen cells of mice with chronic GVHD.

[---●---●---●---] Response of primed splenic T cells of mice with chronic GVHD.

CPM- counts per minute.

Figures in the brackets are stimulation indices.

immunizations with 10^8 killed *S.entertidis*. DTH and lymphoproliferative response to the sonicate of *M. vaccae* and *S. entertidis* were tested one week later.

Normal F1 mice showed excellent DTH and lymphoproliferative response to the sonicates of both *M.vaccae* and *S.entertidis*. As seen earlier, mice with chronic GVHD of 3 weeks duration failed to mount both DTH and lymphoproliferative responses to *M.vaccae* with 3 consecutive immunizations. In contrast, the mice receiving two immunizations with *M.vaccae* and the third immunization with *S.entertidis* showed normal DTH and proliferative response to *S.entertidis* sonicate indicating that the loss of responsiveness observed was specific towards *M. vaccae* sonicate (Table I.3).

c) Antibody response

In order to check whether the observed loss of T-h cell mediated response towards *M.vaccae* antigen affected humoral responses as well, T dependent antibody responses against the immunodominant epitope of *M. vaccae*, the 65- kDa antigen, were also assayed.

Normal F1 mice, after a single i.d. immunization with *M.vaccae* showed mean antibody titre 550 AU/ml on day 7 , which rose to 5835 AU/ml with 3 repeated i.d. immunizations given at one week interval. Mice with chronic GVHD of 3 weeks duration showed a comparable mean anti-65-kDa titre 7 days after a single immunization. On two further immunizations titre did rise to 1,558 AU/ml which was practically $1/4^{\text{th}}$ of the titre observed in normal F1 mice (Table I.3).

Table I.3

DTH, lymphoproliferative and antibody responses against 65-kDa antigen of *M.vaccae*, of thrice immunized (B6 x C3H)F1 mice, normal and those with chronic GVHD of 3 weeks duration.

Mice	DTH CPE± SE	Proliferative response CPM ± SE (SI)		Anti 65-kDa antibody response AU/ML
		Sonicate	65-kDa	
Normal (B6x C3H)F1 3 i.d immunzs. With <i>M.vaccae</i>	0.29 ± 0.01	15,128 ± 1,300 (18.0)	13,207±2,644 (21.0)	5,835±73
Chronic GVHD 3 weeks 3 i.d.immunzs. with <i>M.vaccae</i>	0.09 ± 0.03	3,548 ± 589 (3.3)	1,551 ± 304 (2.9)	1,558±57
Normal (B6x C3H)F1 2 i.d. immnzs. With <i>M.vaccae</i> + 1 i.d. immzn with <i>S.entri</i> .*	0.22 ± 0.02	5,027 ± 325 (11.8)		
Chronic GVHD 3 weeks 2 i.d.immnzns. with <i>M.vaccae</i> + 1 i.d. with <i>S.entri</i> .*	0.20 ± 0.02	5,517 ± 899 (2.5)		

(B6 x C3H)F1 mice, normal and those with chronic GVHD of 3 weeks duration were given three i.d. immunizations with killed *M.vaccae* at one week intervals. One week after the third immunization, DTH and proliferative responses against sonicate/65-kDa antigen and antibody response against the 65-kDa antigen of the organism tested.

*These mice received two i.d. immunizations with killed *M.vaccae* followed by one i.d. immunization with killed *S.enteritidis*. One week later, DTH and proliferative responses against sonicate of both the organisms tested.

SE - Standard error

SI - Stimulation index = mean CPM with Ag/ mean CPM without Ag.

CPE - Corrected foot pad enlargement. CPM - Counts per minute.

Thus based on these results, it was concluded that repeated immunization of mice with chronic GVHD of 3 weeks duration results into antigen specific Th cell non responsiveness.

I.4 Effect of treatment with Cyclosporin A on antigen specific Th cell unresponsiveness induced by repeated immunization of mice with chronic GVHD of only 3 weeks duration

Cyclosporin A (CSA), is known to inhibit T cell activation. The drug inhibits T cell responses to an antigen if given continuously over a long period. In contrast, pulse treatment with CSA at the time of immunization causes modulation of T cell response, instead of total inhibition of the response (512). Effect of pulse treatment with CSA on the Th cell non-responsiveness induced by repeated immunization of mice with chronic GVHD of 3 weeks duration was investigated.

CSA injectible (Sandimmune, Sandoz India) diluted in sterile PBS - 0.5 mg/mouse, was injected intraperitoneally into normal (B6 x C3H)F1 mice and those having chronic GVHD of 3 weeks duration, on 5 consecutive days 0, 1, 2, 3 and 4. On day 1, all mice were immunized i.d. with killed *M.vaccinae*. After 10 days, the 5 day treatment of CSA (reduced dose ---0.25mg/mouse) and i.d. immunization was repeated twice at 10 day interval. Another set of normal (B6 x C3H)F1 mice and those with chronic GVHD of 3 weeks were given 3 repeated i.d. immunization without giving a CSA treatment. One week after the 3rd immunization, the DTH and lymphoproliferative responses were assayed.

As before, repeatedly immunized mice with chronic GVHD of 3 weeks showed poor DTH and lymphoproliferative responses. In

Table I.4

DTH and lymphoproliferative responses of thrice immunized (B6 x C3H)F1 mice, normal and those with chronic GVHD of 3 weeks duration : effect of pulse treatment with Cyclosporine A.

Mice	CSA treatment	DTH CPE (mm) $\pm$ SE	Proliferative response CPM $\pm$ SE (SI)
Normal (B6 x C3H)F1	No	0.29 ± 0.01	$10,441 \pm 713$ (6.9)
Normal (B6 x C3H)F1	Yes	0.65 ± 0.10	$11,137 \pm 1,434$ (17.9)
Chronic GVHD 3 weeks	No	0.09 ± 0.02	$4,149 \pm 205$ (1.8)
Chronic GVHD 3 weeks	Yes	0.63 ± 0.03	$10,189 \pm 928$ (14.6)

Two sets of (B6 x C3H)F1 mice (5 per group), normal and those with chronic GVHD of 3 weeks duration, were given 5 i.p. injections of cyclosporine A (CSA-0.5 mg/mouse in 0.5 ml PBS) on days 0,1,2,3 and 4. Another set received only PBS. All mice were given i.d. immunization with killed *M.vaccæ*. CSA treatment with immunization schedule was repeated twice more at 10 days intervals. One week after the last immunization, the DTH and the proliferative responses of the mice against *M.vaccæ* sonicate were tested as before.

SE - Standard error

SI - Stimulation index = mean CPM with Ag/ mean CPM without Ag.

CPE - Corrected foot pad enlargement. CPM - Counts per minute.

contrast, mice given pulse treatment with CSA along with immunization did mount good DTH and lymphoproliferative response comparable to those of normal F1 (Table I.4).

Based on these results, it was concluded that unresponsiveness emerging in mice with chronic GVHD of 3 weeks duration due to repeated immunization, could be prevented by CSA treatment and hence was related to activation of T cell clones recognizing the antigen namely *M.vacccae*.

I.5 Lymphokine profiles of activated Th cells of test mice with chronic GVHD

a) Lymphokines secreted by the effector T cells of repeatedly immunized mice with chronic GVHD of 3 weeks duration.

Normal (B6 x C3H)F1 and those with chronic GVHD of 3 weeks duration were repeatedly immunized with *M.vacccae* by i.d. route. After 7 days, their DTH test were done to confirm that the mice with chronic GVHD had lost responsiveness to *M.vacccae*. The mice were then sacrificed and the spleen cells were cultured with *M.vacccae* sonicate for 48 hours, after which the supernatants were harvested and assayed for IL-2, IL-4 and IL-10 by ELISA, employing commercial kits from Pharmingen and Genzyme.

Spleen cells of normal mice, even of those with repeated immunization, generated only IL-2. In contrast, mice with chronic GVHD did not generate either IL-2 or IL-4. Significant amounts of IL-4 and IL-10 were present in the culture supernatants of chronic GVHD spleen cells even without antigenic stimulation (Table I.5). The addition of antigen did not induce the Th2 type of profile in the

Table I.5

Profiles of lymphokines secreted by the effectors generated by immunization with *M.vaccae*, of normal (B6 x C3H)F1 mice and those with chronic GVHD of 3 weeks.

	Cytokine (pg/10 ⁶ spleen cells)					
	IL-2		IL-4		IL-10	
	-Ag	+Ag	-Ag	+Ag	-Ag	+Ag
Chronic GVHD 3 weeks with 3 i.d immunzs	0	0	70	68	204	419
Normal (B6x C3H)F1 with 3 i.d. immunzs.	0	70	0	0	0	0
Normal (B6 x C3H)F1 with 1 i.d. immunz.	0	61	0	0	0	0

10⁶ spleen cells, of normal (B6 x C3H)F1 once i.d. immunized with *M.vaccae* , of normal (B x C3H)F1 and of mice with chronic GVHD of three weeks duration thrice immunized i.d. with *M.vaccae* were cultured with 20µg/ml *M.vaccae* sonicate for 48h and their supernates harvested and tested for lymphokine levels by commercial ELISA kits.



culture supernatants as high amounts of IL-4 and IL-10 were present in the supernatants of cultures without the antigen. These results suggest that repeated immunization of mice with chronic GVHD causes loss of responsiveness, rather than Th1 to Th2 shift.

b) Lymphokine secretion by spleen cells of mice with chronic GVHD of 3 months duration induced by polyclonal mitogen Con A

Normal (B6 x C3H)F1 and those with chronic GVHD of 3 months duration were sacrificed and the spleen cells cultured for 72 hours with Con A (5 µg/ml) and supernatants harvested and assayed for IL-2, IL-4 and IL-10. Normal F1 spleen cells produced only IL-2, spleen cells of mice with chronic GVHD of 3 months duration produced much larger amounts of IL-2, IL-4 and IL-10 on stimulation with Con A. The lymphokine profile of normal F1 spleen cells resembled that of naive T cells whereas the profile of cells of mice with chronic GVHD of 3 months duration resembled that of activated / memory T cells (Table I.6).

Thus based on the observations reported so far, it can be concluded that antigen specific non-responsiveness of CD4 + Th cells seen in mice with chronic GVHD was due to activation of T cells. A possible mechanism by which this can happen is by activation induced clonal loss due to apoptosis (333,339). Treatment of mice with strong superantigen such as Staphylococcus enterotoxin B (SEB) has also been shown to induce apoptosis of the Vβ8 T cell clones and thus cause unresponsiveness of the animals T cells to the superantigen (319,331). Hence, extent of unresponsiveness induced by SEB treatment was investigated in the mice with chronic GVHD of 3 weeks duration.

Table I.6

Profiles of lymphokine secretion induced by Con A stimulation of spleen cells of normal (B x C3H)F1 mice and those with chronic GVHD of 3 months duration.

Mice	Cytokine (pg/10 ⁶ spleen cells)					
	IL-2		IL-4		IL-10	
	- Con A	+Con A	-Con A	+Con A	- Con A	+ Con A
Normal (B6 x C3H)F1	0	61	0	0	0	0
Chronic GVHD of 3 months	69	244	0	74	0	384

(B6 x C3H)F1 mice, normal and those with chronic GVHD of three months duration, were sacrificed, spleens removed and single cell suspensions made. Spleen cells were cultured (5 x 10⁵ cells/well) in microtitre plates for 48 h with Con A (5µg/ml), supernatants harvested and assayed for IL-2, IL-4 and IL-10 by ELISA.

I.6 *In vitro* SEB induced proliferative responses of spleen cells of SEB treated mice with chronic GVHD of 3 weeks duration

SEB (Sigma ,5 mg lyophilised) was reconstituted with 5 ml distilled water and aliquoted and kept at -40°C. (B6 X C3H)F1 mice, normal and those with chronic GVHD of 3 weeks duration, were injected SEB (25µg/mouse) by the i.p. route. Two weeks later, the mice were sacrificed spleen cells harvested and *in vitro* proliferative response of the cells to different doses of SEB was tested.

As expected the spleen cells of SEB treated mice, with or without chronic GVHD, showed reduced *in vitro* response to SEB. However, decline in the responses of mice with chronic GVHD was far greater. Based on these results, it was concluded that apoptosis due to *in vitro* stimulation of T cell clones with SEB was greater in mice with chronic GVHD than in normal F1 mice (Fig 3).

Activation induced cell death (AICD) due to apoptosis has been generally considered to be a physiological event to control the size of the clonal burst following immunizations and to prevent autoreactive clones from mounting a pathological immune response to self antigens (292,293). AICD due to apoptosis could be the consequence of altered antigen presentation as proposed by Russel (339). Therefor antigen presentation by the irradiated spleen cells of normal mice and those with chronic GVHD of 3 weeks duration was investigated.

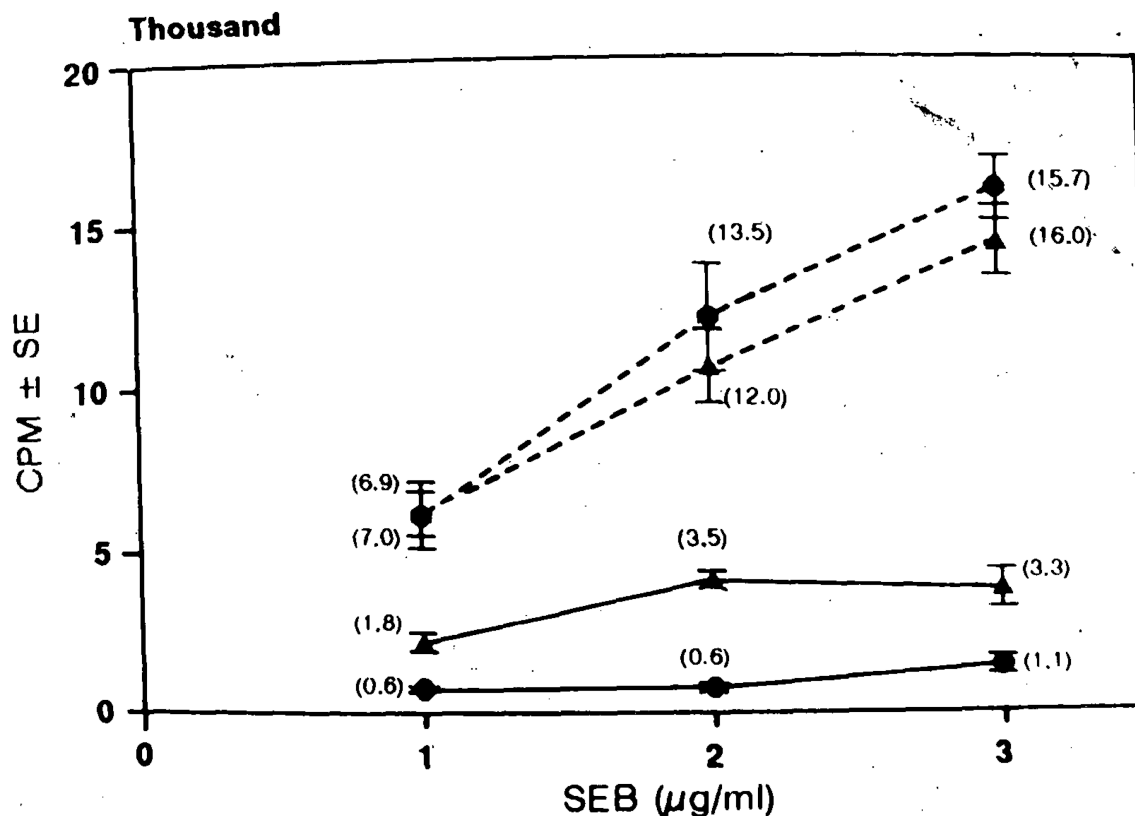


Fig 3 *In-vitro* proliferative responses of spleen cells to SEB : effect of prior *in-vivo* treatment with SEB.

(B6 $\times$ C3H)F1 mice, normal and those with chronic GVHD of three weeks duration were given an i.p. injection of 25 μg /mouse of SEB. The mice were sacrificed 15 days later, the spleens removed and the splenic cells were cultured with SEB and a 72h proliferative assay performed.

[--- Δ --- Δ --- Δ ---] Response of spleen cells of normal untreated mice.

[--- Δ --- Δ --- Δ ---] Response of spleen cells of normal mice treated with SEB.

[--- $\bullet$ --- $\bullet$ --- $\bullet$ ---] Response of spleen cells of untreated mice with chronic GVHD of three weeks duration.

[--- $\bullet$ --- $\bullet$ --- $\bullet$ ---] Response of spleen cells of treated mice with chronic GVHD of three weeks duration.

I.7 Comparison of antigen presentation by spleen cells of mice with and without chronic GVHD

Normal (B6 X C3H) F1 mice were immunized i.d. with *M. vaccae*, after 7 days spleens removed single cell suspension made and T cells separated over nylon wool column. 10^5 i.d primed T cells were cultured with 10^5 irradiated spleen cells from normal F1 mice or those with chronic GVHD to serve as APC's. The cells were stimulated with *M. vaccae* sonicate and a standard lymphoproliferative assay performed.

Proliferative response induced by splenic APC of chronic GVHD was significantly lower i.e. 50% of that induced by splenic APC's of normal F1 mice (Table I.7).

The observed low antigen specific response of primed T cells from normal F1 on antigen being presented by APC's of syngeneic mice with chronic GVHD could be due to two alternative possibilities. Firstly, antigen presentation abnormality of APC's of these mice might lead to inadequate expansion of primed T cells and hence the proliferative response would be low. Alternatively, the APC's of mice with chronic GVHD might stimulate T cell expansion as efficiently as that by APC's of normal F1 mice, however, the former may induce excessive AICD, leading to the observation of low proliferative response. Antigen specific proliferation in the standard proliferative assay was recorded by pulsing with thymidine for 16 hours after 120 hour culture. It is known that peak proliferation induced by antigen, occurs at approximately 48 hours. It was therefor essential to compare the proliferative responses induced at 48h- 72h and 120 h to asses the degree of AICD. However, frequency

Table I.7

Antigen presentation by the splenic and thymic APC's of normal (B6 x C3H)F1 mice and those with chronic GVHD of 3 weeks duration.

Source of APC's	Mice	Proliferative response CPM $\pm$ SE
Spleen cells	Normal (B6 x C3H)F1	22,822 $\pm$ 2,100
Spleen cells	Chronic GVHD of 3 weeks	10,100 $\pm$ 1,389
Thymus cells	Normal (B x C3H)F1	7,642 $\pm$ 861
Thymus cells	Chronic GVHD of 3 weeks	3,643 $\pm$ 98

(B6 x C3H)F1 mice, normal and those with chronic GVHD of 3 weeks duration, were sacrificed, spleens and thymuses removed, single cell suspensions made and subjected to 800 rad γ irradiation, for use as APC's. A separate set of normal (B6 x C3H)F1 mice were given an i.d. immunization with *M.vaccae*, their spleens removed, single cell suspensions made and T cells separated. 10^5 i.d. primed T cells were mixed with equal number of irradiated spleen or thymus cells of normal (B6 x C3H)F1 mice or those with chronic GVHD of 3 weeks. The cells were cultured with *M.vaccae* sonicate and a standard antigen specific lymphoproliferation assay performed.

CPM - counts per minute
SE - standard error



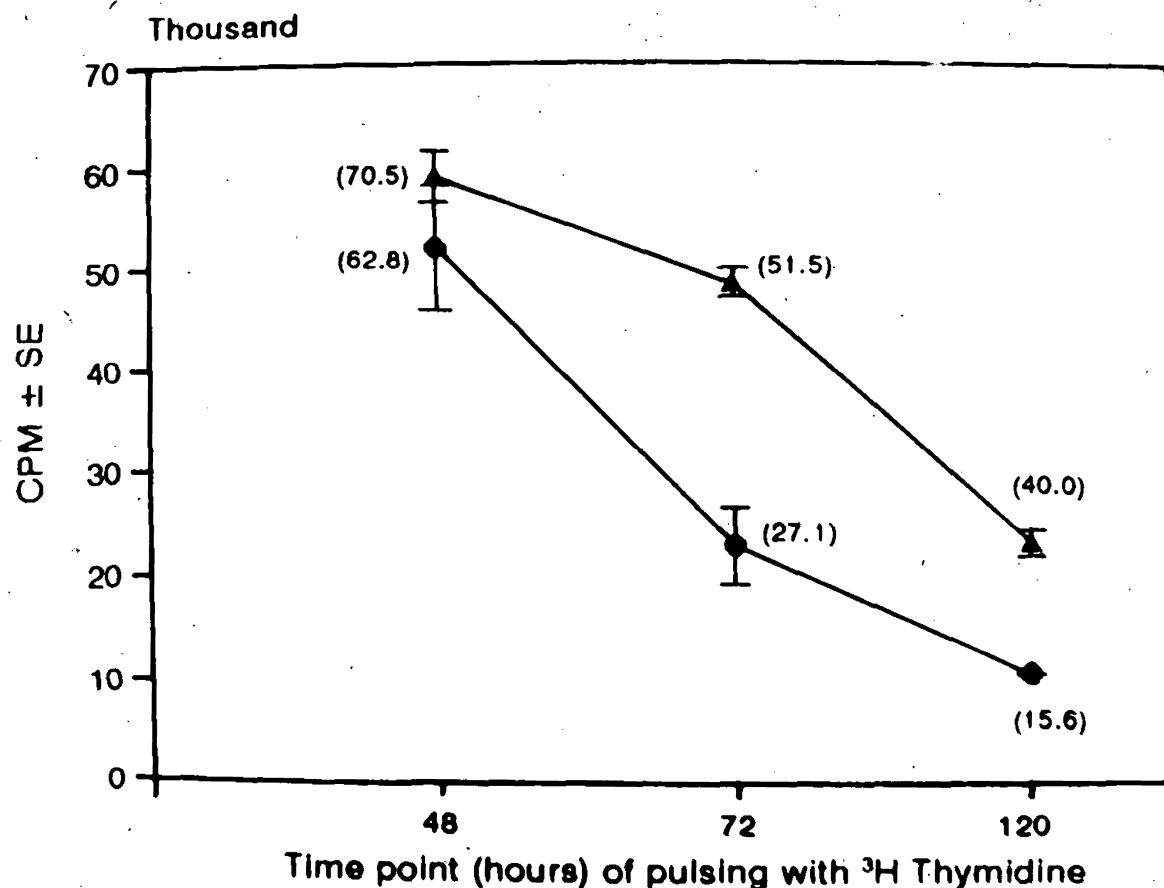


Fig 4 Effect of APCs of mice with chronic GVHD on in-vitro response of normal splenic T cells to Con A.

(B6 × C3H) F1 mice, normal and those with chronic GVHD of three weeks duration were sacrificed, spleens harvested and single cell suspensions made. Part of the cells of normal mice were passed over nylon wool column to obtain T cells. The remaining normal cells and the cells of the mice with chronic GVHD were irradiated or use as APCs. 10^5 normal splenic T cells were mixed with equal number of irradiated spleen cells of normal mice or those with chronic GVHD and cultured with Con A ($5 \mu\text{g}/\text{ml}$). The cultures were pulsed with ^3H thymidine at 48 h, 72 h and 120 h for a duration of 16 h and incorporation of label assayed.

[▲▲▲] Responses of cells cultured with APCs of normal mice.

[●●●] Responses of cells cultured with APCs of mice with chronic GVHD.

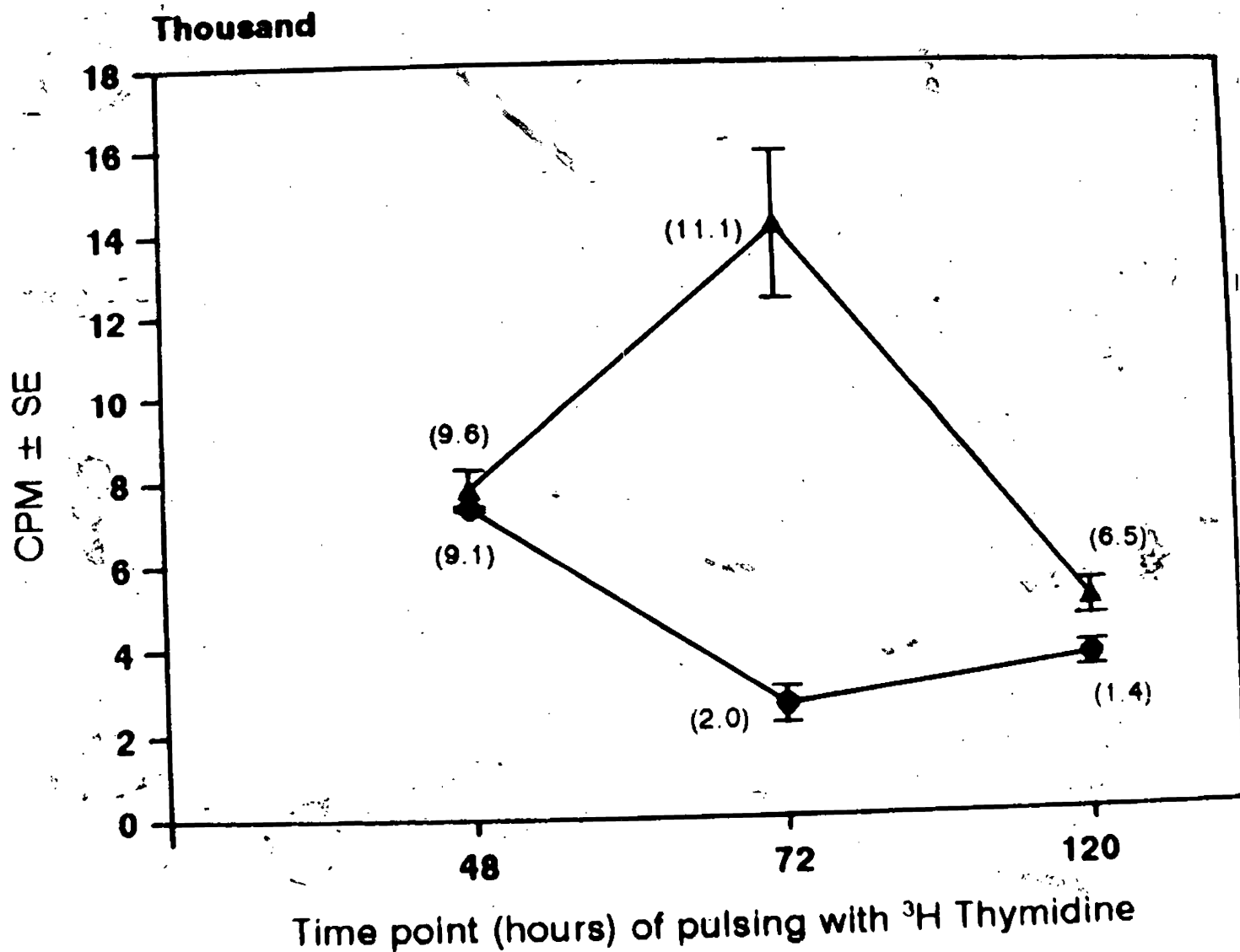


Fig 5 Effect of APCs of mice with chronic GVHD on in-vitro response of normal splenic T cells to SEB.

(B6 × C3H)F1 mice, normal and those with chronic GVHD of three weeks duration were sacrificed, spleens harvested and single cell suspensions made. Part of the cells of normal mice were passed over nylon wool column to obtain T cells. The remaining normal cells and the cells of mice with chronic GVHD were irradiated for use as APCs. 10^5 normal splenic cells of normal mice or those with chronic GVHD and cultured with SEB ($1\mu\text{g}/\text{ml}$). The cultures were pulsed with ^3H thymidine at 48h, 72h and 120h for a duration of 16h and incorporation of label assayed.

[▲ ▲ ▲] Responses of cells cultured with APCs of normal mice.

[● ● ●] Responses of cells cultured with APCs of mice with chronic GVHD.

of antigen specific T cells being low, antigen specific proliferation at 48h- 72h is not recordable in microculture assays. Hence it was decided to investigate this issue with a polyclonal mitogen Con A and a superantigen SEB . Naive T cells were stimulated with these agents in presence of APC's of normal mice and those with chronic GVHD . Proliferation was assayed at three time points 48h, 72h and 120h.

Normal (B6 X C3H) F1 mice were sacrificed, spleens harvested and T cells separated. 10^5 normal T cells were cultured with 10^5 irradiated spleen cells of normal F1 or those with chronic GVHD of 3 weeks duration. The cultures were stimulated with 5 μ g/ml Con A or 1 μ g/ml SEB and pulsed at three different time points 48h, 72h and 120h with 3 H thymidine . These were harvested 16h later and proliferation assayed.

Con A induced proliferation, with normal spleen cells as APC's peaked at 48h, the degree of proliferation was sustained till 72h and declined at 120h possibly due to apoptosis (Fig 4) SEB also induced similar pattern of response showing significant proliferation at 48h which increased further at 72 h and declined thereafter. (Fig 5)

In contrast, with spleen cells of mice with chronic GVHD as APC's the response kinetics was found to be altered. 48h proliferative responses induced by both Con A and SEB were similar to the responses with splenic APC's from normal mice. However thereafter the responses declined steeply, to a level which was 16-50% of that observed with splenic APC's of normal mice.

Thus, peak proliferation induced by APC's of mice with or without chronic GVHD was comparable. However, thereafter the proliferation induced by APC's of mice with chronic GVHD was much lower. Since the difference between peak and late phase proliferation is likely to be due to AICD, it was concluded that the APC's of mice with chronic GVHD cause much more AICD than the APC's of normal mice.

Argument - Emergence of non responsiveness could also be due to fall in the contribution of new, recently matured, T cells to the peripheral lymphoid tissues from the thymus. Since antigen presentation by the APC's in the peripheral lymphoid tissue of mice with chronic GVHD was abnormal and lead to excessive AICD, it is also possible that the thymic stromal APC's of the animals may also behave abnormally. Hence antigen presentation by the thymic APC's of normal F1 mice and mice with chronic GVHD was compared.

I.8 Antigen presentation by thymic cells of normal mice and those with GVHD

Thymocytes were separated from normal F1 and from those with chronic GVHD of 3 weeks duration and irradiated. i.d. primed T cells were separated from normal F1 as before, and 10^5 T cells were cultured with 10^5 irradiated thymocytes to serve as APC's and were stimulated with *M vaccae* sonicate. Antigen presentation by thymus cells was not as efficient as that of splenic cells. Irradiated thymus cells of mice with chronic GVHD, induced a proliferative response that was practically 50% of that induced by normal thymus cells (Table I.7).

PART II

Study of role of Superantigen derived from infectious mouse mammary tumor virus (MMTV) in activation of CD8+ Ts cells by an unrelated bacterial antigen.

Factors which lead to the activation of functionally irrelevant CD8 + T cells capable of abrogating or reducing the size of CD4+ Th cell memory and effector response by competing for antigen and/or lymphokines required for growth and differentiation was investigated by using our earlier reported model of CD8+ T cell mediated suppression.

In our earlier studies (4,41) route of immunization was observed to play a significant role in deciding the T cell response to immunization with a killed mycobacterial vaccine. Since the same model has been utilized in the present study the experimental model is described in detail. Immunization by i.d. route with a killed vaccine of *M.vacccae* generated both DTH and lymphoproliferative responses - the effector and memory responses respectively. In contrast, i.p. immunization failed to generate these. Further investigation on the cause of nonresponsiveness showed that i.p. immunization resulted in activation of both CD4+ and CD8+ T cells and that the CD8+ T cells brought about the suppression of the response of the former. The suppression mediated by these CD8+ T cells could be adoptively transferred to the syngeneic hosts which suppressed the subsequent i.d. immunization with *M.vacccae* (41). Adoptive transfer of spleen cells of mice primed i.p. with *M vacccae*, had no effect on i.d. immunization with a different immunogen such as *S.enteritidis*. The *in vitro* experiments further confirmed the above

observations. Further studies revealed that monoclonal anti-IA antibody could suppress the antigen induced proliferative response of mice primed i.d. with *M.vacccae*. In contrast, monoclonal anti-IE antibody enhanced antigen-specific proliferation of spleen cells primed i.p. with *M.vacccae*. The i.p. primed splenic CD8⁺ T cells when mixed with i.d. primed spleen cells, could also suppress the *in-vitro* antigen induced proliferative response of the latter and the suppression could be blocked by anti-IE antibodies. Thus, the suppression was mediated by CD8⁺ T cells in this experimental model and it was shown to be IE restricted (489). Therefore the cells responsible for suppression were designated as T- suppressor (Ts) cells.

Mechanism of suppression induced by i.p. primed CD8⁺ Ts cells was investigated. The i.p. primed CD8⁺ T cells could compete with simultaneously primed CD4⁺ T cells for IL-2 and thus abrogate the response of the latter (46). This was shown in an *in-vitro* assay in which primed CD4⁺ T cells were stimulated with exogenous IL-2. In case of i.d. primed and non primed spleen cells, cultures stimulated with IL-2 showed excellent proliferation. Addition of 65-kDa antigen of *M.vacccae* had no effect on proliferation of non primed cells. Addition of the antigen to the i.d. primed cells enhanced their proliferation. However, addition of antigen to the i.p. primed cells suppressed their proliferation. It was further shown that the suppression was due to CD8⁺ T cells which could absorb IL-2 in the culture medium on activation by the antigen. Thus the mechanism of suppression induced by CD8⁺ Ts cells of i.p. primed mice was identified to be due to competition with simultaneously primed CD4⁺ Th cells for IL-2. It was important to note that in this model CD8⁺ Ts cells were expanded only on i.p. immunization and not on i.d. immunization. Further for efficient *in vitro* competition,

the CD8⁺ Ts cells had to be reactivated *in vitro*. This technique of *in vitro* competition for IL-2 in a proliferative assay could be employed to investigate *in vitro* activation of CD8⁺ Ts cells.

Generation of suppression on i.p. immunization was studied in various inbred strains and was shown that IE negative strains did not generate suppression. However, *in vitro* experiments with i.p. primed cells of IE negative strains such as C57BL/6 employing the technique for *in vitro* activation of CD8⁺ Ts cells, showed unexpected results; they did show expansion of CD8⁺ Ts cells on i.p. immunization.

Recognition of the 65-kDa antigen by the CD8⁺ Ts cells was also investigated employing the above technique. The recognition appeared to be promiscuous, the cells recognized the antigen on IA, IE or even allogeneic IE, even though IE was the preferred MHC. These lead to a conclusion that the recognition of antigen by CD8⁺ Ts cells was IE dependent and not restricted.

IE dependent recognition and recognition of antigen presented on allogeneic IE are features of T cell - superantigen interactions (58,492,493). This raises the obvious question as to if the 65-kDa antigen recognized by CD8⁺ Ts cells is a superantigen. The observation that i.d. immunization does not activate CD8⁺ Ts cells also seemed to implicate a superantigen but of mouse mammary tumor viral (MMTV) origin. The dendritic cells, which are the main APC's in the dermis and skin do not express MMTV superantigen (495). In contrast, in the peritonium there are B cells which are known to express the superantigen of MMTV.

Thus we were confronted with the following obvious question -

Is 65-kDa antigen of *M.vacccae* a superantigen ? If not

Is MMTV superantigen involved in the activation of CD8⁺ Ts cells when immunization is done by the i.p. route ?

In case this is true, what is the role of the superantigen in the activation of CD8⁺ Ts cells by the 65-kDa antigen of *M.vacccae*.

Since a processed antigen and a superantigen interact with different domains of MHC - class II and TCR, Janeway has proposed that a superantigen can enhance a low affinity interaction of a class II presented peptide antigen with TCR (497). It was therefore decided to investigate this possibility in activation of CD8⁺ Ts cells in our experimental model.

The question of CD8⁺ Ts cell activation and expansion on i.p. immunization was investigated in the context of expansion of IE and a MMTV derived superantigen. For this, we have employed four strains of mice C3H, B6 and two sets of F1 mice (C3H Female X B6 Male) F1 and (B6 Female X C3H Male) F1. C3H mice are IE positive and are chronic carriers of infectious MMTV. B6 strain is IE negative and does not carry MMTV infection. (C3H Female X B6 Male) F1 are like C3H mothers i.e. they express IE and are chronic MMTV carriers as virus is maternally transmitted through milk (513) . (B6 Female X C3H Male) F1 express IE but do not have MMTV infection.

II.1 DTH and lymphoproliferative responses of C3H, B6 and the two sets of F1 mice on i.d. and i.p. immunization with *M.vacccae*.

C3H, B6 and the two sets of F1 mice were immunized by i.d. or i.p. route with 10⁸ killed *M. vacccae* and their DTH responses to the sonicate or the 65-kDa antigen tested one week later. Primed mice

were also sacrificed and their lymphoproliferative responses to the above antigens tested.

All the four strains showed a good DTH response to i.d. immunization. Response to i.p. immunization depended upon the strains. C3H mice failed to show a DTH response whereas the B6 mice showed a definite DTH response, though the degree of foot pad swelling was less than that of the i.d. primed B6. Response of the F1 strains depended upon the maternal parent. (C3H × B6) F1 failed to mount a DTH response on i.p. immunization, whereas (B6 × C3H) F1 showed a definite response (Table II.1).

The lymphoproliferative responses too showed a similar pattern. The i.d. primed spleen cells of all the four strains showed excellent proliferation on stimulation with the sonicate or the 65-kDa antigen of *M.vaccæ*. As with DTH, the proliferative response of the i.p. primed spleen cells, also depended upon the strain. The C3H spleen cells showed a poor response, whereas the B6 cells showed a good response. Again, response of the spleen cells of the F1 strains depended upon the maternal parent. (C3H × B6) F1 spleen cell's proliferative response was poor where as the response of (B6 × C3H) F1 spleen cells was good (Table II.2).

Thus failure to mount DTH and lymphoproliferative responses was observed only in mice immunized by the i.p. route and only in strains expressing IE and having chronic MMTV infection.

The observed non responsiveness to immunization by the i.p. route, seen in C3H and (C3H × B6) F1 mice could either be due to tolerisation or antigen specific suppression. The i.p. primed C3H spleen cells were, therefore treated with anti-Ly 2+ antibody and

Table II.1

DTH responses of mice immunized with *M.vaccae* by i.d. or i.p. route.

Strain	IE	MMTV ^{c)}	CPE (mm) ± SE		
			Route of immunization		
			i.d.	i.p.	i.p. followed by i.d. ^{a)}
C3H	+	+	0.29 ± 0.02	0.02 ± 0.01	0.03 ± 0.01
C3H ^{b)}	+	+	0.26 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
C3H	+	+	0.28 ± 0.02	0.03 ± 0.01	0.31 ± 0.02 ^{d)}
C57Bl/6	-	-	0.36 ± 0.03	0.29 ± 0.01	0.27 ± 0.01
(B6 x C3H)F1	+	-	0.33 ± 0.01	0.21 ± 0.01	0.25 ± 0.01
(C3H x B6)F1	+	+	0.22 ± 0.01	0.08 ± 0.02	0.05 ± 0.02

Mice (5/group) were immunized with 10⁸ killed *M.vaccae* cells by i.d./i.p. route and 1 week later their DTH response to the sonicate tested.

- a) Mice were given an i.p. immunization with 10⁸ killed *M.vaccae*; 1 week later the same mice were given a second immunization with the same number of organisms by the i.d. route and their response tested.
- b) DTH responses of a separate set of C3H mice, immunized by i.d. route were also tested with 65-kDa antigen of *M.vaccae* (1.0µg/footpad).
- c) Productive MMTV infection
- d) Mice were given an i.p. immunization with *M.vaccae*, followed 1 week later with an i.d. immunization with *S.enteritidis* and DTH response to latter tested 1 week later.

CPE - corrected foot pad enlargement.
SE- standard error.

complement to deplete CD8⁺ T cells, and a lymphoproliferative assay performed with the sonicate of *M.vaccæ*. Removal of CD8⁺ T cells enhanced the proliferation of the i.p. primed cells. This was thought to be in favour of the latter possibility (Table II.2).

The i.d. and i.p. primed C3H cells were also cultured with 65-kDa antigen of *M.vaccæ* and anti-IA and anti-IE antibodies respectively, and a lymphoproliferative assay performed. Anti-IA antibody added to the cultures, inhibited proliferation of both i.d. and i.p. primed cells, whereas the anti-IE antibodies enhanced the proliferation of i.p. primed cells and had no effect on i.d. primed cells (Table II.2). Based on these results, the possibility of antigen specific proliferation (CD4⁺ Th response) and suppression (? CD8⁺ Ts response) being respectively, IA and IE restricted/dependent, was considered very likely.

II.2 Non responsiveness to i.p. immunization is due to activation of CD8⁺ T suppressors.

a) Immunization with killed *M.vaccæ* by the i.p. route abrogates the ability of C3H and (C3H × B6) F1 mice to respond to subsequent i.d. immunization with the same organism.

In order to investigate if the observed non responsiveness on i.p. immunization of C3H and (C3H × B6) F1 mice is due to activation of suppressors, all the four strains were given 10⁸ *M.vaccæ* by the i.p. route, followed 1 week later by a second immunization by the i.d. route and their DTH responses tested. C3H and (C3H × B6) F1 failed to mount a DTH response, whereas the B6 and (B6 × C3H) F1 showed no such effect. This effect of i.p. immunization on subsequent i.d. immunization was antigen specific. Immunization by the i.p. route with *M.vaccæ* had no effect on subsequent i.d. immunization with

Table II.2

Lymphoproliferative responses of mice primed with *M.vaccae* by i.d. or i.p. route.

Route of priming	Antigen ^a	Antibody ^b	MEAN CPM ± SE (SI)			
			C3H	B6	(C3HxB6)F1	(B6xC3H)F1
i.d.	sonicate	-	12,350 ± 908 (8.2)	1475 ±1,116 (9.9)	16,340 ± 1,880 (10.9)	12,852 ± 1,462 (8.6)
i.p.	sonicate	-	3,910 ± 502 (2.6)	9,998 ± 482 (6.8)	4,205 ± 869 (6.8)	9,390 ± 714 (6.3)
i.p. ^c	sonicate	-	9,438 ± 874 (6.7)	ND	ND	ND
i.d.	NCM-sonicate	-	15,621 ±1,365 (12.1)	ND	ND	ND
i.d.	NCM-65-kDA	-	14,493 ±1,350 (11.4)	ND	ND	ND
i.d.	NCM-65-kDA	Anti-IA	3,150 ± 375 (4.3)	ND	ND	ND
i.p.	NCM-65-kDA	Anti-IE	9,000 ± 715 (6.1)	ND	ND	ND
i.d.	NCM-65-kDA	Anti-IE	12,510 ±1,046 (10.6)	ND	ND	ND

The four strains were primed with killed *M.vaccae* by i.d. /i.p. route as before and 1 week later they were sacrificed, spleen cells harvested and a standard antigen specific proliferative assay performed.

- For stimulation, sonicate (20µg/ml) or NCM adsorbed sonicate or 65-kDa antigen of *M.vaccae* were employed.
- Neat supernatants of hybridoma cultures of anti-Ia^k or anti-IE (all alleles) antibodies were added to cultures (20µl/culture).
- Anti-Ly2⁺ complement treated spleen cells of mice primed i.p. with *M.vaccae*

SE - standard error

CPM - counts per minute

SI - Stimulation index = mean CPM with Ag/mean CPM without Ag.

Salmonella enteritidis. It was therefore concluded that i.p. immunization of C3H and (C3H × B6) F1 mice generated antigen specific non responsiveness, that could abrogate the response to subsequent i.d. immunization. No such effect was seen with B6 and (B6 × C3H) F1 mice (Table II.1).

b) Non responsiveness can be adoptively transferred by i.p. primed splenic CD8⁺ T cells ; Both cellular and humoral responses are abrogated.

C3H mice were primed by the i.p. route as before, one week later they were sacrificed, spleens harvested single cell suspensions made and transferred to syngeneic hosts. The recipients were then immunized with *M.vaccae* by the i.d. route and their DTH responses tested one week later. They failed to mount a DTH response. The recipients were then sacrificed, spleens harvested, single cell suspension made and a lymphoproliferative assay performed with sonicate or the 65-kDa antigen. The recipients of i.p. primed cells failed to mount a proliferative response. A separate set of 10 C3H mice, given i.p. primed syngeneic cells, were immunized i.d. with *M.vaccae*, tested for DTH as before and the absence confirmed. They were then bled from the retro orbital plexus and their antibody response against the 65-kDa antigen of *M.vaccae* assayed by ELISA. The response of mice receiving i.p. primed cells was considerably lower than that observed in simultaneously immunized control mice which did not receive i.p. primed cells. Thus both cellular and humoral responses were suppressed by adoptive transfer of i.p. primed cells (Table II.3). Therefore, it was concluded that the phenomenon was truly "suppression", and the cells responsible for the phenomenon the "suppressor cells". The "suppressors" generated by i.p. immunization with *M.vaccae* did not abrogate the DTH and antigen specific proliferative responses of the recipients to

Table II.3

Adoptive transfer of suppression by spleen cells of mice primed i.p. with *M.vaccae* : Phenotypic markers of "T-suppressors".

Expt No.	Treatment given to the i.p. primed cells	Responses of the recipients to i.d. immunization		
		DTH CPE $\pm$ SE	Proliferation CPM $\pm$ SE (SI)	Anti 65-kDa antibody AU/ml
1.	Non primed cells given to the recipients	0.28 ± 0.01	$12,650 \pm 545$ (9.6)	501 ± 39
2.	None	0.04 ± 0.01	$4,123 \pm 327$ (3.1)	80 ± 3
3. *	None	0.29 ± 0.01	$7,321 \pm 357$ (9.3)	ND
4.	Anti-Thy Ab + complement	0.30 ± 0.02	ND	ND
5.	Anti-Ly2 Ab + complement	0.34 ± 0.03	$19,540 \pm 895$ (14.1)	ND
6.	Anti-L3T4 Ab + complement	0.03 ± 0.01	$3,150 \pm 345$ (3.2)	ND
7.	Anti-L3T4 Ab + complement reated, i.d. primed cells	0.32 ± 0.02	$13,510 \pm 635$ (9.4)	ND

C3H mice were immunized with 10^8 killed *M.vaccae* by the i.p. route, spleen cells harvested 1 week later and used as such or after treatment with anti-Thy, anti-Ly2 or anti L3T4 antibody and complement. 5×10^7 such cells were transferred to the syngeneic recipients (8 per group), who were then given an i.d. immunization with *M.vaccae*. The DTH, spleen cell proliferation and the serum antibody responses against 65-kDa antigen, of the recipients were tested one week later.

*In experiment no.3, the recipients were given spleen cells of mice primed i.p. with *M.vaccae*. They were then immunized i.d. with *S.enteritidis* and their DTH and spleen cell proliferation tested 1 week later against the sonicate of the latter organism.

In experiment No.7, the recipients were given anti-L3T4 treated spleen cells of mice primed i.d. (instead of i.p.) with *M.vaccae*.

SE - Standard error

SI - Stimulation index = mean CPM with Ag/ mean CPM without Ag.

CPE - Corrected foot pad enlargement. CPM - Counts per minute.

an i.d. immunization with *S.enteritidis* (Table II.3), indicating their antigen specificity.

Experiments were also done to identify the phenotypic markers on the suppressor cells. The i.p. primed spleen cells of C3H mice were treated with anti-Thy, anti-L3T4 or anti-Ly2 antibody and complement to deplete cells of the respective phenotype. The so treated spleen cells were transferred to syngeneic hosts. The recipients were then immunized with *M.vaccac* by the i.d. route and 1 week later their DTH and lymphoproliferative responses tested. Ability to induce suppression on adoptive transfer was eliminated on treatment of cells with either anti-Thy or anti-Ly2 antibody, implying that the suppressors were Ly2⁺ (CD8⁺) T cells (Table II.3).

c) Immunization by i.d. route fails to generate suppression.

Since all the four strains mounted a DTH and a lymphoproliferative response on immunization by the i.d. route , it was presumed that suppressors are not stimulated on immunization by this route. It can be argued that CD8⁺ T cells were also expanded, but not in numbers adequate to abrogate the *in vivo* response.

To test this possibility, the C3H mice were immunized by i.d. route as before, sacrificed 1 week later, spleens harvested and single cell suspension made. These were treated with anti-L3T4 antibody and complement to remove DTH effectors. Such CD8 enriched 5×10^7 cells were adoptively transferred to syngeneic hosts, who were then immunized by the i.d. route with *M vacccac* and their DTH and lymphoproliferative responses tested 1 week later. The mice showed no evidence of immune suppression, suggesting that i.d. priming did not stimulate suppressors (Table II.3).

d) i.p. primed CD8⁺ T cells co-cultured with i.d. primed cells suppress in vitro antigen induced proliferation of i.d. primed cells.

Separate sets of C3H mice were primed i.p. and i.d. with *M vaccae* as before, sacrificed one week later, spleens harvested and single cell suspensions made. The i.p. primed cells were passed over a nylon wool column to obtain T cells which were then treated with anti-L3T4 antibody and complement to obtain i.p. primed CD8⁺ T cells. 2×10^5 i.d. primed spleen cells were mixed 5×10^4 i.p. primed CD8⁺ T cells and cultured with the 65-kDa antigen of *M vaccae*, with and without anti-IE antibody and a lymphoproliferation assay performed. The proliferative response of i.d. primed cells was suppressed by the splenic CD8⁺ T cells of the i.p. primed mice and the suppression was blocked significantly by the anti-IE antibody (Table II.4).

Based on these results, it was concluded that immunization with killed *M vaccae* by i.p. route only, induced specific immune suppression ; i.d. immunization did not. The suppression was due to activation of *M vaccae* specific CD8⁺ T suppressors . These results were based on the studies of C3H mice only. Therefore similar kind of studies were undertaken on B6, and two sets of F1 mice as described below

II.3 Adoptive transfer of suppression with i.p. primed spleen cells of B6 and the two sets of F1 mice.

Studies of DTH and lymphoproliferative responses following i.p. immunization seemed to indicate that B6 and (B6 x C3H) F1 did not generated suppression, whereas C3H and (C3H x B6) F1 did. Whether these findings correlated with expansion of CD8⁺ Ts cells

Table II.4

Suppression of in-vitro proliferation of i.d. primed spleen cells by the i.p. primed CD8⁺ T cells.

No. i.p. primed cells added to i.d. primed cells	Antibody	Proliferative response of i.d. primed spleen cells CPM $\pm$ SE (SI)
5 x 10 ⁴ non primed CD8 ⁺ T cells	-	12,322 $\pm$ 560 (9.2)
5 x 10 ⁴ i.p. primed CD8 ⁺ T cells	-	4,357 $\pm$ 325 (2.9)
5 x 10 ⁴ i.p. primed CD8 ⁺ T cells	Anti-IE	8,412 $\pm$ 613 (6.2)
5 x 10 ⁴ i.p. primed CD4 ⁺ T cells	-	15,312 $\pm$ 875 (11.7)

C3H mice were immunized with 10⁸ killed *M.vaccae* by the i.p. route, spleen cells harvested 1 week later, passed over nylon wool column and then treated with anti-L3T4 or anti-Ly2 antibody and complement to obtain i.p. primed CD8⁺ and CD4⁺ T cells respectively. A separate set of C3H mice were immunized i.d. with *M.vaccae* and spleen cells harvested 1 week later. 2 x 10⁵ i.d. primed spleen cells were mixed with 5 x 10⁴ non primed or i.p. primed CD8⁺ or CD4⁺ T cells as shown in column 1. The cells were cultured with 65-kDa antigen of *M.vaccae* and a standard lymphoproliferation assay performed.

SE - Standard error

SI - Stimulation index = mean CPM with Ag/ mean CPM without Ag.

CPM - Counts per minute.

or lack of CD8⁺Ts cells in these strains was therefore investigated using adoptive transfer experiments.

Mice of all four strains were given an i.p. immunization with *M vaccae* . One week later their spleen cells were harvested and transferred to the syngeneic recipients . In case of F1 strains, their i.p. primed spleen cells were also transferred to the parental strains. Adoptive transfer of primed cells was done with whole spleen cells or spleen cells treated with anti-L3T4 or anti-Ly2 antibody and complement to deplete cells of respective phenotype. All the recipients were given an i.d. immunization with *M vaccae* and their DTH responses tested one week later.

The cells of i.p. primed (C3H × B6) F1 mice behaved like the cells of C3H mice, i.e. they abrogated the DTH response of the syngeneic recipients. Therefore it was concluded that in (C3H × B6) F1 mice also, CD8⁺ Ts cells were expanded on i.p. immunization. In contrast, i.p. primed spleen cells of B6 and (B6 × C3H) F1 mice failed to induce suppression on adoptive transfer into syngeneic recipients (Table II.5). Based on these results , one should have concluded that i.p. priming did not expand CD8⁺ Ts cells in these strains. However, the following observations indicate that the phenomenon is much more complex.

Effects of adoptive transfer of i.p. primed F1 cells on responses of parental C3H and B6 strain mice to i.d. immunization were quite unexpected.

(C3H × B6) F1 cells, which could abrogate the responses of syngeneic recipients, abrogated the response of C3H mice also but

Table II.5

Adoptive transfer of suppression by i.p. primed spleen cells of (C3H x B6)F1, B6 and (B6 x C3H)F1 strains of mice.

Source of spleen cells ^{a)} given before i.d. immunization	CPE (mm) $\pm$ SE of recipients ^{b)}			
	C3H	B6	(B6 C3H)F1	(C3HxB6)F1
None	0.28 $\pm$ 0.01	0.36 $\pm$ 0.03	0.32 $\pm$ 0.02	0.25 $\pm$ 0.02
Anti-L3T4 treated i.p. primed, of (C3H x B6)F1	0.06 $\pm$ 0.01	0.32 $\pm$ 0.03	0.09 $\pm$ 0.02	0.10 $\pm$ 0.01
Anti-L3T4 treated i.p. primed, of B6	-	0.33 $\pm$ 0.02	-	-
Anti-L3T4 treated i.p. primed, of (B6 x C3H)F1	0.10 $\pm$ 0.01	0.33 $\pm$ 0.02	0.32 $\pm$ 0.03	0.13 $\pm$ 0.02
Anti-Ly2 treated i.p. primed, of (B6 x C3H)F1	0.32 $\pm$ 0.02	ND	ND	ND

a) The indicated strains of mice were given an i.p. immunization with 10^8 killed *M.vaccae*; 1 week later the spleen cells were harvested and treated as shown and then transferred by tail vein injection to different strains shown.

b) The recipients of i.p. primed cells were immunized with 10^8 killed *M.vaccae* by i.d., route and their DTH response tested 1 week later.

SE - Standard error

CPE - Corrected foot pad enlargements

not of B6 mice (Table II.5). This indicated the requirement of IE expression for induction of effective *in vivo* suppression.

Spleen cells of i.p. primed (B6 X C3H) F1 mice showed unusual behavior in adoptive transfer experiments. Even on enrichment for CD8⁺ Ts cells, they were ineffective in abrogating the responses of syngeneic or parental B6 recipients, to i.d. immunization with *M vaccae*. Yet, the same cell preparation effectively suppressed the responses of C3H mice, which are MMTV infected (Table II.5).

In view of the above findings , effect of adoptive transfer of i.p. primed spleen cells of the two F1 strains into each other was also investigated. As expected, transfer of i.p. primed (C3H x B6) F1 cells abrogated the response of (B6 x C3H) F1 mice. Surprisingly, the i.p. primed (B6 x C3H) F1 cells also, on adoptive transfer, abrogated the response of (C3H x B6) F1 mice to i.d. immunization, even though such an adoptive transfer was ineffective in supporting the response of the syngeneic mice . Further , transfer of non primed (B6 x C3H) F1 cells into (C3H x B6) F1 mice had no effect on the responses of the recipients to i.d. immunization , indicated the necessity of i.p. priming (Table II.5).

From the above results, it is obvious that it would be incorrect to conclude that i.p. priming of (B6 x C3H) F1 mice does not expand CD8⁺ Ts cells, just because - (i) the mice responded to i.p. immunization with a DTH and a lymphoproliferative response and (ii) their i.p. primed spleen cells could not abrogate the response of syngeneic recipients to i.d. immunization. Difference in the behavior of the two sets of F1 mice to i.p. immunization could be due to difference in the size of the CD8⁺Ts cell pool expanded following i.p.

immunization, it being greater in (C3HxB6) F1 mice and therefore effective in abrogating the response .

Earlier we have reported (46) that antigen specific CD8+ T “suppressor” cell compete with primed CD4+ Th cells for exogenously added IL-2 in a proliferative assay and as a consequence proliferation of CD4+ T cells is inhibited. For efficient competition, the CD8+ T cells have to be reactivated *in vitro* by the antigen. Therefore the *in vitro* assay for suppressors described by us, was employed to provide a definitive proof that even in B6 and (B6 x C3H) F1 strains i.p. immunization resulted in expansion of CD8+ Ts cells, though their DTH and lymphoproliferative responses were not abrogated.

For the purpose of understanding, the earlier reported assay has been described in detail which has been taken from our published report.

II.4 *In vitro* assay of suppressor function of i.p. primed CD8+ T cells : Exogenously added IL-2 induced proliferation of unprimed, i.d. and i.p. primed spleen cells, and addition of antigen induced suppression of proliferation of i.p. primed cells.

C3H mice, unprimed or primed 1 week earlier by the i.d. or i.p. route with a vaccine containing 10^8 killed *M.vaccae*, were sacrificed. Their spleens were removed and 2×10^5 spleen cells were cultured in 200 μ l medium having different amounts of IL-2 containing Con A sup in presence or absence of 65-kDa antigen of *M.vaccae* and their proliferation assayed.

Con A sup induced excellent proliferation of all the three types of cells, i.e. unprimed, i.d. and i.p. primed. Addition of antigen had no effect on unprimed cells, and caused some enhancement in the proliferation of i.d. primed cells. However, addition of antigen to the i.p. primed cells caused profound suppression of proliferation. Removal of CD4⁺ T cells from the three types of cell preparations by antibody and complement, abrogated proliferation of all the three, induced by Con A sup. Removal of CD8⁺ T cells from the i.p. primed spleen cells abrogated the antigen induced suppression of proliferation. The Con A sup induced proliferation could be blocked by anti-IL-2 antibody. Based on these data it can be concluded that the predominant growth factor in the Con A sup, causing proliferation was IL-2; the proliferating cells were CD4⁺ T cells, not necessarily specific for *M.vaccae*. Further i.p. priming alone resulted into expansion of CD8⁺ T cells capable of suppressing the proliferation of CD4⁺ T cells, induced by IL-2 in the Con A sup. The suppression was observed only on stimulation with antigen, the 65-kDa molecule, the same antigen, as that recognized by Th cells responsible for DTH and lymphoproliferative responses of mice primed i.d. with *M vaccae*. Earlier it has been reported from our laboratory (46) that i.p. primed CD8⁺ T cells can bind and absorb IL-2 from Con A when restimulated in-vitro with the 65-kDa antigen. Thus the above assay can be used as an in-vitro assay for CD8⁺ T cell activation (Fig 6).

It was decided to employ the above *in vitro* assay for the CD8⁺ T cells, to investigate if the antigen specific 'suppressors' were expanded on i.p. immunization of B6 and (B6 x C3H) F1 strains.

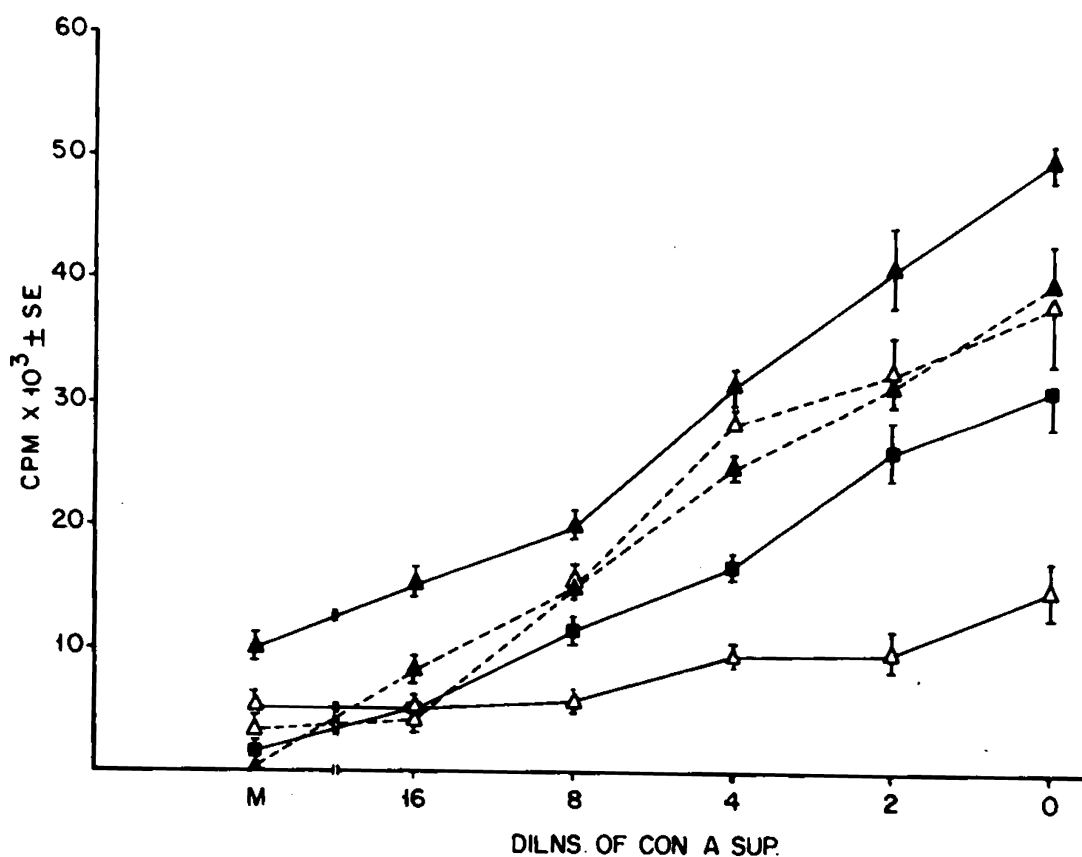


Fig 6. IL-2 induced proliferation of spleen cells of mice, non-primed or primed i.d. or i.p. with *M.vaccae*.

C3H mice, non-primed or primed i.d. or i.p. with 108 killed *M.vaccae*, 1 week earlier, were killed and spleen cell suspensions made. Cells were cultured in the medium containing different dilutions of Con A sup (IL-2 content 540 IU/ml) in presence or absence of 65-kDa antigen of *M.vaccae* for 136h, and their proliferation assayed by incorporation of ³H thymidine.

[■] Non-primed cells ; [▲] i.d. primed cells; [△] i.p. primed cells;

[—] cells cultured with 65-kDa antigen; [----] cells cultured without antigen.

M : medium only.

II.5 B6 and (B6 x C3H) F1 mice immunized by i.p. route show evidence of expansion of CD8+ Ts cells in the *in vitro* assay.

The mice of the two strains were given i.p. or i.d. immunization, and one week later their spleen cells cultured with Con A sup and 65-kDa antigen, to check for evidence of suppression. As with C3H mice, both i.d. and i.p. primed cells of these strains too showed excellent IL-2 induced proliferation; addition of antigen had no suppressive effect on i.d. primed cells. However, i.p. primed cells of both these strains also showed suppression of proliferation on addition of antigen. As with C3H mice, even here the suppression could be abrogated by removal of CD8+ T cells (Table II.6) . Thus, even though mice of these two strains did mount an *in vivo* DTH response on i.p. immunization, their spleen cells did show evidence of expansion of CD8+ Ts in the *in vitro* assay.

As we had observed that addition of anti-IE could block the suppression brought about by the CD8+ T cells, MHC restriction of CD8+ T cells was investigated in these strains of mice. A single source of i.p. primed CD4+ and CD8+ T cells were cultured with APC's derived from different source and restriction investigated with anti-IA and anti-IE antisera.

II.6 Study of MHC restriction of activation of CD8+ Ts cells

[This is duplication of work published earlier (46). It has been repeated because of its relevance to the present objective of investigation of the role of MMTV superantigen in CD8+ Ts cell activation.]

Splenic T cells (10^5) of i.p. primed (C3H x B6) F1 mice were mixed with 10^5 irradiated naive (C3H x B6) F1 or C3H or B6 spleen

Table II.6

IL-2 induced proliferation of unprimed, i.d. and i.p. primed spleen cells of B6 and (B6 x C3H)F1 : Antigen induced suppression of proliferation of i.p. primed cells.

NO	Cells	Antigen	Mean CPM $\pm$ SE
1.	Non primed spleen cells of B6 mice	-	39,518 $\pm$ 5,859
2.	Non primed spleen cells of B6 mice	+	38,733 $\pm$ 3,418
3.	i.d. primed spleen cells of B6 mice	-	43,137 $\pm$ 2,450
4.	i.d. primed spleen cells of B6 mice	+	67,598 $\pm$ 5,878
5.	i.p. primed spleen cells of B6 mice	-	51,309 $\pm$ 3,023
6.	i.p. primed spleen cells of B6 mice	+	8,907 $\pm$ 203
7.	non primed spleen cells of (B6 x C3H)F1	-	53,719 $\pm$ 2,754
8.	non primed spleen cells of (B6 x C3H) F1	+	45,462 $\pm$ 1,705
9.	i.d. primed spleen cells of (B6 x C3H)F1	-	51,243 $\pm$ 1,381
10.	i.d. primed spleen cells of (B6 x C3H)F1	+	71,627 $\pm$ 15,432
11.	i.p. primed spleen cells of (B6 x C3H)F1	-	57,836 $\pm$ 10,321
12.	i.p. primed spleen cells of (B6 x C3H)F1	+	9,222 $\pm$ 831
13.	Anti-Ly2 + complement treated, i.p. primed spleen cells of (B6 x C3H)F1	+	50,524 $\pm$ 10,108

B6 and (B6 x C3H)F1 mice, nonprimed or primed i.d. or i.p. with 10^8 killed *M vaccae* 1 week earlier, were sacrificed and spleen cell suspension made. The Con A sup (IL-2 - 270 IU/ml) and 65-kDa antigen of *M vaccae* were added in the wells and cultured for 136 h and their proliferation assayed.

SE - standard error

CPM- counts per minute

cells to serve as APC's, and cultured in Con A sup (IL-2 - 270-IU/ml) with 65-kDa antigen. Anti-Ia^b, anti-Ia^k or anti-IE antibodies were added to block suppression.

When antigen was presented by (C3H x B6) F1 or C3H APC's, only anti-IE blocked suppression; anti-IA^b and anti-Ia^k antibodies were ineffective. When the same pool of (C3H x B6) F1 i.p. primed T cells were cultured with B6 APC's, the suppression was blocked by anti-IA^b.

These findings imply that whenever the APC's expressed IE, the recognition by CD8⁺ Ts cells was only IE mediated. When APC's expressed only IA (as in B6 cells), the same T cell pool showed IA mediated recognition. These observations are in favor of the conclusion that antigen recognition by the CD8⁺ Ts cells lacked rigid restriction specificity usually imposed by MHC (Table II.7).

Similar experiments with i.p. primed B6 T cells seemed to confirm these conclusions. When the antigen was presented by B6 APC's, the suppression was blocked by anti-IA^b antibodies; with (C3H x B6) F1 cells as APC's the suppression was blocked by both anti-IA^b and anti-IE antibodies; blocking effect of both the antibodies on suppression was partial (Table II.7). Since B6 animals do not express IE, the recognition of antigen by their CD8⁺ Ts cells on IE can be truly considered as recognition mediated by allogeneic MHC class II.

Thus the observation supports the conclusion that recognition of 65-kDa antigen by the CD8⁺Ts cells lacked rigid MHC imposed restriction specificity and it was MHC class II dependent and not restricted, preferred class II being IE.

Table II.7

MHC restriction of Ts generated in B6 and (C3H x B6)F1 mice by i.p. immunization with M.vaccae.

No	Source of i.p. primed cells	Source of APC	Antigen	Anti-Ia Antibody	Mean CPM $\pm$ SE
1.	(C3H xB6) F1	(C3H xB6) F1	-	-	19,065 $\pm$ 3,023
2.	(C3H xB6) F1	(C3H xB6) F1	+	-	1,596 $\pm$ 396
3.	(C3H xB6) F1	(C3H xB6) F1	+	anti-Ia ^b	1,146 $\pm$ 233
4.	(C3H xB6) F1	(C3H xB6) F1	+	anti-Ia ^k	1,432 $\pm$ 446
5.	(C3H xB6) F1	(C3H xB6) F1	+	anti-IE	15,992 $\pm$ 1,303
6.	(C3H xB6) F1	C3H	+	anti-Ia ^k	823 $\pm$ 65
7.	(C3H xB6) F1	C3H	+	anti-IE	16,292 $\pm$ 2,759
8.	(C3H xB6) F1	B6	+	anti-Ia ^b	16,546 $\pm$ 914
9.	B6	B6	-	-	75,108 $\pm$ 933
10.	B6	B6	+	-	8,912 $\pm$ 188
11.	B6	B6	+	anti-Ia ^b	25,290 $\pm$ 1,705
12.	B6	(C3H xB6) F1	-	-	67,698 $\pm$ 5,878
13.	B6	(C3H xB6) F1	+	-	2,132 $\pm$ 547
14.	B6	(C3H xB6) F1	+	anti-Ia ^b	24,964 $\pm$ 1,579
15.	B6	(C3H xB6) F1	+	anti-IE	24,241 $\pm$ 1,042

(C3H x B6)F1 and B6 mice were immunized with M vaccae by the i.p. route. One week later their spleens removed, and the cell suspension passed over nylon wool column to obtain T cells. I.p. primed T cells (10^5) were mixed with 10^5 different naïve irradiated whole spleen cells to serve as APC, and cultured with Con A sup (IL-2- 270-IU/ml) and the 65-kDa antigen of M vaccae. Neat hybridoma fluid (20 μ l) of the required anti-Ia specificity was added to the cultures, incubated for 120h, pulsed with ³H thymidine for 16h and incorporation of lable assayed.

It is known that superantigen interactions behave in this fashion, i.e. 1) There is no rigid MHC imposed restriction specificity (58,493,492). 2) Interaction occurs even with APC's expressing allogeneic MHC (497). 3) Most superantigen interactions prefer IE as the class II specificity (58).

Thus , activation of CD8+Ts cells by the 65-kDa antigen of *M.vaccae* appeared to be similar to activation of T cells by a superantigen. Therefore, the possibility of the 65-kDa antigen of *M.vaccae* being a superantigen, was investigated.

II.7 65-kDa antigen of *M.vaccae* does not behave like a superantigen in C8+T cell activation.

Class II (IE) dependence of CD8+ Ts cells recognition of 65-kDa antigen, indicate the likelihood of 65-kDa antigen being a superantigen. A superantigen interacts with MHC class II and TCR V β on their lateral domains. It is known that superantigens do not need to be processed for being recognized by T cells. Hence effect of 0.1M chloroquin and 10mM Ammonium chloride, agents inhibiting processing by the class II (endosomal) pathway, on the *in vitro* proliferation of the i.d. primed C3H spleen cells induced by the 65-kDa antigen was investigated. No proliferation was observed in cultures containing these agents. Superantigens are also polyclonal mitogens to T cells expressing a particular V β specificity. Hence naive C3H spleen cells were cultured with varying amounts of 65 kDa antigen of *M vacccae* and a 72h proliferation assay performed. The antigen was not mitogenic to naive T cells (data not shown).

Based on these results, it was concluded that 65-kDa of *M.vacccae* did not behave as a superantigen.

II.8 (B6 x C3H) F1 mice can be induced to mount specific suppression on immunization by the i.p. route by transfusion of non immune irradiated C3H or (C3H x B6) F1 cells prior to i.p. immunization.

(B6 x C3H) F1 mice were given intravenously 5×10^7 irradiated normal spleen cells of C3H or (C3H x B6) F1 mice. This was immediately followed by an i.p. immunization with *M. vaccae* and 7 days later their DTH response tested. As controls, similarly treated mice given i.d. immunization and untreated mice given i.p. immunization were also tested. Recipients of irradiated C3H or (C3H x B6) F1 cells showed suppression on i.p. immunization, whereas the control mice did not. Immunization by i.d. route was unaffected by such a cell transfer prior to immunization (Table II.8).

Based on these results it was concluded that C3H or (C3H x B6) F1 cells provided some factor that contributed to IE dependent activation and expansion of CD8⁺ T cells to numbers adequate to suppress *M.vacccae* specific CD4⁺ Th response on i.p. immunization. It may be noted that the putative factor had no effect on i.d. immunization.

An IE dependent factor involved in expansion of T cells can be a superantigen. Since C3H and (C3H x B6) F1 mice are chronic carriers of MMTV, the involved superantigen could be the ORF protein of MMTV. Activation of T cells by the ORF superantigen of MMTV (C3H) is known to be mediated by its interactions with V β 14⁺ T cell receptors (TCR) and IE on the APC (513,514,515). Therefore,

Table II.8

Transfer of irradiated naïve C3H or (C3H x B6)F1 spleen cells induces (B6 x C3H)F1 mice to mount suppression on i.p. immunization.

Cells given before immunization	Route of immunization	CPE (mm) $\pm$ SE
None	i.d.	0.37 $\pm$ 0.02
None	i.p.	0.31 $\pm$ 0.02
5 x 10 ⁷ irradiated normal C3H spleen cells	i.p.	0.10 $\pm$ 0.02
5 x 10 ⁷ irradiated normal C3H spleen cells	i.d.	0.38 $\pm$ 0.02
5 x 10 ⁷ irradiated normal (C3H x B6)F1 spleen cells	i.p.	0.05 $\pm$ 0.01
5 x 10 ⁷ irradiated normal (C3H x B6)F1 spleen cells	i.d.	0.37 $\pm$ 0.02
5 x 10 ⁷ irradiated normal (C3H x B6)F1 spleen cells	i.p. followed by i.d.	0.09 $\pm$ 0.02

(B6 x C3H)F1 mice were given by tail vein injection irradiated spleen cells of normal C3H or (C3H x B6)F1 mice. 24h later, the recipients were immunized with *M.vaccæ* by the i.p. or i.d. route, and their DTH response tested 1 week later. In the last experiment, the recipients were first given an i.p. immunization followed 1 week later by an i.d. immunization and the DTH tested 1 week later.

SE - Standard error

CPE - Corrected foot pad enlargement

such an activation can be blocked if either of these interactions are blocked.

II.9 Anti-V β 14 antibody can not block suppression induced by CD8+ T cells of i.p. primed mice.

C3H mice were given an i.p. immunization with *M.vaccae* and 1 week later their spleens removed, single cell suspension made and cultured with 65-kDa antigen of *M.vaccae* and a standard antigen specific lymphoproliferation assay performed. Effect of anti-IE and anti-V β 14 antibodies on the proliferative response was tested. Contrary to our expectation the anti-V β 14 antibody did not enhance the proliferation (i.e. did not block suppression). As before, anti-IE antibody blocked the suppression (Table II.9). Being an anti-TCR antibody, the anti-V β 14 antibody can activate the T cells by TCR modulation. Therefore, the possibility that the antibody in fact activated the V β 14+ Ts cells, instead of blocking them, was considered very likely.

II.10 Anti-V β 14 antibody induces proliferation of naive cells ; causes suppression of 65-kDa specific proliferation of i.d. primed cells

2×10^5 naive normal C3H spleen cells were cultured with 100 μ l of neat hybridoma fluid of anti-V β 14 specificity and a 72 h proliferation assay was performed. The antibody induced proliferation of naive spleen cells (mean CPM 6938 ± 347 , SI 4.6), indicating that the antibody could activate the V β 14+ T cells by modulating their TCR.

Table II.9

Effect of anti-IA, anti-IE and anti-V β 14 antibodies on the *in-vitro* proliferative responses of spleen cells of mice primed i.d. or i.p. with *M.vaccae*.

Route of priming	Antibody	MEAN CPM $\pm$ SE (SI)	
		65-kDa	Con A
i.d.	-	12,350 $\pm$ 908 (8.2)	39,578 $\pm$ 2,381 (26)
i.d.	anti-IA	3,150 $\pm$ 375 (4.3)	ND
i.d.	anti-IE	11,312 $\pm$ 763 (8.0)	ND
i.d.	anti-V β 14	6,525 $\pm$ 380 (4.1)	35,849 $\pm$ 1,269 (23)
i.p.	-	3,910 $\pm$ 502 (2.6)	ND
i.p.	anti-IE	9,348 $\pm$ 515 (6.9)	ND
i.p.	anti-V β 14	4,375 $\pm$ 510 (2.9)	ND

C3H mice were primed with killed *M.vaccae* by i.d. or i.p. route, one week later a standard lymphoproliferative assay performed on the primed spleen cells with 65-kDa antigen of *M.vaccae* or Con A as the stimulating ligand. Neat hybridoma fluid of anti-IA or anti-IE or anti-V β 14 specificity (20 μ l/culture well) was added to the cultures.

SE - standard error

SI - Stimulation index = mean CPM with ligand/ mean CPM without ligand.

Both *in vivo* and *in-vitro* experiments indicate that the CD8⁺ T cells are not stimulated when mice are primed by the i.d. route with *M. vaccae*. Possible explanation is that the dermal APCs do not express the MMTV superantigen required for CD8⁺ T cell activation. It is therefore logical to ask if anti-V β 14 antibody which can activate V β 14⁺ T cells, can in fact induce V β 14⁺ CD8⁺ T cells of i.d. primed mice to recognize class II presented 65-kDa antigen and cause antigen specific suppression. Hence the effect of anti-V β 14 antibody on the *in-vitro* proliferation of i.d. primed (not i.p. primed) spleen cells was investigated. As before, spleen cells of mice primed i.d. with *M.vaccae* were cultured with the 65-kDa antigen of the organism and a standard 6 day proliferation assay performed in presence or absence of the anti-V β 14 antibody. Antigen induced excellent proliferation and addition of anti-V β 14 antibody (which was mitogenic to naive spleen cells) caused significant suppression of proliferation induced by the 65-kDa antigen (Table II.9). In order to investigate the specificity of suppression, effect of anti-V β 14 antibody on the Con A induced *in vitro* proliferative response of the same cell suspension, was also studied. The cells were cultured with Con A (5 μ g/ml) and the antibody and a 72h proliferation assay performed. Anti-V β 14 antibody had no effect on Con A induced proliferation (Table II.9).

Based on these data, it was concluded that activation of V β 14⁺ T cells by TCR modulation with anti-V β 14 antibody, may have caused suppression of proliferation of i.d. primed T cells induced by the 65kDa antigen. This was considered to be indirect evidence suggesting that the "suppressors" were activated by anti-V β 14 antibody and the 65-kDa antigen. In this assay we could not be unequivocally certain of the specificity that the V β 14⁺ T cells were activated both by the 65-kDa antigen and the anti-V β 14 antibody.

To achieve this objective, we needed an in-vitro assay for CD8+ T cell activation. The assay of CD8+ T cell activation described in section 4 was employed.

II.11 Simultaneous stimulation by both 65-kDa antigen and anti-V β 14 antibody induces suppression of IL-2 induced proliferation of i.d. primed spleen cells.

2×10^5 i.d. primed C3H spleen cells were cultured with Con A sup (IL-2 content 270-IU/ml) and stimulated with anti-V β 14 antibody and 65-kDa antigen and their proliferation assayed as before.

Addition of Con A sup induced excellent proliferation of i.d. primed cells; addition of antigen or anti-V β 14 antibody alone had no effect on the proliferation. However, addition of both anti-V β 14 antibody and 65-kDa antigen caused significant suppression of proliferation of i.d. primed cells induced by Con A sup. This may be compared with results in section 4-6; for suppressors of IL-2 induced proliferation of i.p. primed cells, 65-kDa antigen alone was adequate (Table II.10).

II.12 Intraperitoneal priming of most IE expressing mouse strains induces suppression.

In our earlier studies, several IE + strains were shown to generate suppression on i.p. immunization (46,489). In these strains, endogenous MMTV derived *mtv* coded superantigens could play a role similar to MMTV superantigen in C3H and (C3H $\times$ B6) F1. Therefore, ability to generate suppression on i.p. immunization with *M.vacccae*, was investigated in several strains and compared with reported *mtv* derived superantigen expression and consequent V β

Table II.10

**IL-2 induced proliferation of i.d. primed spleen cells of C3H mice :
anti-V β 14 and 65-kDa antigen induce suppression of proliferation.**

No.	Antigen	Anti-V β 14 antibody	MEAN CPM $\pm$ SE
1.	-	-	29,968 $\pm$ 5,514
2.	+	-	33,158 $\pm$ 5,371
3.	-	+	28,253 $\pm$ 5,337
4.	+	+	11,343 $\pm$ 2,501

C3H mice were immunized with 10^8 M vaccae by the i.d. route. One week later, the mice were sacrificed, and their spleen cells were cultured with Con A sup (IL-2-270-IU/ml). The cultures also received 65-kDa antigen of M vaccae and 20 μ l of neat hybridoma fluid of anti-V β 14 specificity as indicated in the table. The cultures were incubated for 120h, pulsed with 3 H thymidine for 16h and incorporation of the lable assayed. The cells gave a mean CPM of 3,559 $\pm$ 351, when cultured with no exogenous IL-2.

deletion in these strains (513,514,515). BALB/c, DBA/2, (B6 x BALB/c) F1 and out bred Swiss white mice were immunized i.d. or i.p. with *M.vaccae* and their DTH response studied one week later. In all these strains i.d. immunization induced a DTH response whereas the i.p. immunization did not. Further, i.p. priming of mice of these strains abrogated their ability to mount a subsequent response by the i.d. route. Based on this data it was concluded that all these strains showed specific suppression on i.p. immunization and it could not be cor-related with expression or non-expression of known *mtv* coded superantigens associated with theses strains of mice (Table II.11).

Table II.11

DTH responses of mice immunized with *M.vaccae* by i.d. or i.p. route.

STRAIN	MTV ^{c)}	MMTV ^{d)}	CPE (mm) $\pm$ SE		
			Route of immunization		
			i.d.	i.p.	i.p. followed by i.d. ^{a)}
C3H ^{b)}	1,6,8,11, 14	MMTV (C3H)	0.26 $\pm$ 0.01	0.02 $\pm$ 0.01	0.03 $\pm$ 0.01
C3H ^{c)}			0.28 $\pm$ 0.02	0.03 $\pm$ 0.01	0.31 $\pm$ 0.02
B6	8,9,17	-	0.36 $\pm$ 0.03	0.29 $\pm$ 0.01	0.27 $\pm$ 0.01
(B6 xC3H)F1	1,6,8,9,11 14, 17	-	0.33 $\pm$ 0.01	0.21 $\pm$ 0.01	0.25 $\pm$ 0.01
(C3H x B6)F1	1,6,8,9,11 14,17	MMTV (C3H)	0.22 $\pm$ 0.01	0.08 $\pm$ 0.02	0.05 $\pm$ 0.02
Balb/C3H	6, 8, 9	MMTV (SW)	0.28 $\pm$ 0.01	0.08 $\pm$ 0.02	0.02 $\pm$ 0.01
(B6 X Balb)F1	6, 8, 9, 17	-	0.29 $\pm$ 0.01	0.08 $\pm$ 0.01	0.06 $\pm$ 0.01
DBA/2	1,6,7,8,11 1314,17	-	0.28 $\pm$ 0.02	0.07 $\pm$ 0.02	0.06 $\pm$ 0.02
Swiss white	-	MMTV (SW)	0.29 $\pm$ 0.02	0.05 $\pm$ 0.02	0.03 $\pm$ 0.01

Mice (5/group) were immunized with 10⁸ killed *M.vaccae* by i.d./i.p. route, and 1 week later their DTH response to the sonicate tested.

a) Mice were given an i.p. immunization with 10⁸ killed *M.vaccae*, 1 week later the same mice were given a second immunization with the same number of organisms by the i.d. route and their response tested.

b) DTH responses of a separate set of C3H mice, immunized by i.d. route were also tested with 65-kDa antigen of *M.vaccae* (1.0 μ g/footpad), results were similar.

c) *Mtv* genomes integrated in the germ line ().

d) Exogenous productive virus

e) Mice were given an i.p. immunization with *M.vaccae*, followed 1 week later with an i.d. immunization with *S.enteritidis* and DTH response to latter tested 1 week later.

CPE - corrected foot pad enlargement.
error

SE - Standard

DISCUSSION



In the present study two different models of T cell activation are investigated. In both the models, the ultimate outcome is non responsiveness of CD4⁺ T cells. In the first model repeated immunization of mice with chronic GVHD results into specific non responsiveness of CD4⁺ T cells. In the second model, intraperitoneal immunization results into activation of CD8⁺ T suppressor cells, capable of competing with simultaneously activated CD4⁺ Th cells for growth factors, such as IL-2 and thus causing antigen specific non-responsiveness of CD4⁺ Th cells. In the first model, i.e. induction of antigen specific non responsiveness, in chronic GVHD, the mechanism of induction has been investigated. In the second model, the mechanism of induction of non responsiveness has already been worked out. The so called CD8⁺ T suppressor cells (Ts) compete with simultaneously primed CD4⁺ Th cells for IL-2 (46). However, the mode of activation of CD8⁺ Ts cells by class II presented bacterial antigen has evaded logical explanation; this issue has been investigated in the present study.

I - Immunological abnormalities in chronic GVHD.

Chronic GVHD is a complex chronic disease induced by injection of allogeneic spleen or lymph node cells in a host. For a successful induction, the host has to be tolerant to the donor cells, without being immunocompromised (56). The disease process is due to a complex cascade of events triggered by sustained activation of donor CD4⁺ T cells by host MHC class II alloantigens (56,475). Functionally active host immune system is also essential for induction of chronic GVHD (56,515). The disease has characteristic immune abnormalities. In the early stages of the disease, the T cells of the of the host are unable to respond to the self MHC restricted foreign

antigens, however the T cell response to allogeneic MHC and polyclonal mitogens is relatively unaffected (57,485). In the late stages of chronic GVHD total non responsiveness is observed. Despite this abnormality in T cell responsiveness, the animals have antibodies to a variety of self antigens and tissues, including RNA, DNA erythrocytes etc (474). The experimental/clinical chronic GVHD is associated with autoimmune disorders such as SLE also (474,480). The SLE like syndrome of chronic GVHD is not induced in an irradiated host indicating that the intact host immune system is a prerequisite (56,474,516). Further the experimental animals/patients of chronic GVHD show evidence of lymphoid hyperplasia, as indicated by splenomegaly and lymphadenopathy (474,517). Thus the above observations are suggestive of the fact that activation of host's lymphoid system is also an important event in the pathogenesis of this syndrome. The findings in chronic GVHD have two contrasting features. Chronic lymphoid hyperplasia and autoantibodies indicate a sustained, possibly polyclonal, activation of lymphoid cells. In contrast, emergence of T cell non responsiveness to foreign antigens, with polyclonal response being relatively unaffected, is suggestive of T cell clonal loss. Immunological changes observed in HIV infection also show such qualitative CD4⁺ Th cell defects. Shearer and colleagues (479) drew attention to the similarities between murine chronic GVHD and AIDS and suggested that murine chronic GVHD may be an experimental model to study pathogenesis in HIV infection.

Thus, the chronic GVHD could be a model of activation induced clonal loss. The phenomenon, if confirmed, would be distinct from immune tolerance, because the latter is antigen specific whereas the nonresponsiveness observed in chronic GVHD is general.

In our experimental model of chronic GVHD, which was induced by injecting parental C3H cells into (B6 x C3H)F1 mice, marked splenomegaly with GVH indices ranging from 1.34 to 4.4 was seen. Animals showed autoantibodies against erythrocytes and generation of antibodies against ds DNA. These chronic GVHD animals when examined 3 months after inducing GVHD, showed T cell nonresponsiveness to a randomly selected immunogen - *M. vaccae* vaccine. The animals were unable to mount a DTH and a proliferative response. However, the response to polyclonal mitogen Con A was relatively unaffected.

These results did indicate that the experimental model employed by us, is a classical model of chronic GVHD with typical immunological abnormalities as reported by others (57,479,480,485).

The chronic GVHD animals of only 3 weeks duration did not show the immune defect; the mice could mount DTH, lymphoproliferative and antibody responses against the 65-kDa antigen of *M. vaccae*. Thus it was obvious to investigate the events occurring between 3 weeks and 3 months which might have lead to the classical immunological abnormality.

In order to understand whether the observed nonresponsiveness was related to activation of the specific lymphoid clones, the effect of repeated immunization in chronic GVHD of 3 weeks duration was investigated. With 3 repeated immunizations with *M. vaccae* a total nonresponsiveness to the immunogen was observed in these animals. All the CD4+ T helper mediated responses were low. The animals showed poor DTH and proliferative

responses which progressively declined with each immunization. Antibody titre against 65-kDa antigen was also low in comparison with that in normal F1 mice with three repeated immunizations. The observed nonresponsiveness was found to be specific against *M. vaccae* as response to *S. enteritidis* was not affected. As there was actual reduction in antibody titre against 65-kDa antigen, the observed nonresponsiveness could not be argued to be a Th1 to Th2 shift.

It was necessary to study the causes that induced the abnormality in the activation resulting into nonresponsiveness towards a specific antigen after repeated immunization and to confirm whether the effect seen was in fact related to activation of CD4⁺ T cells. The use of any suppressant to suppress the activation of specific clones recognizing *M. vaccae*, would also affect the induction of GVHD. Therefore there was a need for an experimental design that uses an immunosuppressant in such a way that it influences antigen induced activation of T cells of F1 mice without affecting GVH induction in a major way.

Cyclosporin A (CsA), the classical T cell immunosuppressant, can also be used as an immunomodulator. Bretscher and Havele (512), reported an interesting observation that pulse therapy with CsA can switch the immune response induced by antigen from a humoral to a cell-mediated mode. By changing the concentration and the course of the drug, they reported the peculiar findings that a particular response such as Th1/Th2 can be inhibited or enhanced by pulse therapy with certain concentration of the drug when given under conditions that would normally allow antigen to induce such a response, in the absence of the drug.

Hence, a pulse therapy of CsA was given, to both normal F1 and to those with chronic GVHD of 3 weeks duration, with each immunization. The ability to mount an immune response was enhanced as seen by greater foot pad enlargements in normal F1. In chronic GVHD, the antigen induced non-responsiveness (DTH and lymphoproliferative responses) was shown to be abrogated. Thus it was concluded that the phenomenon of non-responsiveness observed in chronic GVHD truly reflects activation induced phenomenon.

As a parallel to tolerance, the antigen induced non-responsiveness could be due to anergy or activation induced clonal loss due to apoptosis. As apoptosis is a physiological mechanism that regulates the size of the clonal expansion (292,293,523), the non-responsiveness observed due to activation was quite paradoxical.

Pathology of chronic GVHD involves activation of parental T cells by alloantigens of the other parent on F1 cells; this may affect the normal physiological process of antigen induced activation. Hence a formal proof was essential for the hypothesis that activation of T cell clones in mice with chronic GVHD in fact caused pathologically excessive apoptosis. The aspect of AICD was investigated by using a known *in vivo* model of AICD caused by a superantigen.

Administration of a single dose of a strong superantigen such as SEB, has been reported to cause loss by apoptosis of V β 8.1 and 8.2 positive T cell clones, capable of interacting with the superantigen (319,331). Hence the behavior of chronic GVHD mice on treatment with SEB was investigated in comparison with that of normal F1. Lymphoid cells of both the groups of mice, normal and those with chronic GVHD, on treatment with SEB showed depletion in their

ability to respond to the superantigen *in vitro*, apparently due to clonal loss. However, the extent of loss of responsiveness to SEB was observed to be more in case of chronic GVHD indicating a greater loss of SEB reacting clones in mice with chronic GVHD. Hence it was concluded that the degree of activation induced apoptosis was greater in mice with chronic GVHD.

One of the reasons for T cells to succumb to PCD could be abnormal antigen presentation, which has been reported in HIV infection also. Chronic GVHD generates large amounts of cytokines, produced by T donor cells which can bring about changes in antigen presenting cells. Hence it was decided to ask whether antigen presentation by APCs of GVH mice differs from that of normal.

i.d. primed splenic T cells were stimulated *in-vitro* with the *M.vaccae* antigen presented by irradiated naive spleen cells of normal F1 or of those with chronic GVHD. As speculated the response generated by splenic APCs of mice with chronic GVHD was very poor. It is known that peak proliferation of activated T cells occurs at 24-48 hrs after activation. Hence single point determination of proliferative response may not be able to prove the presentation abnormality resulting into excessive clonal loss due to apoptosis. Therefore it was necessary to examine proliferation at different time points after activation.

As the frequency of antigen recognizing cells is too low at 48 h in the microculture assay, it is not possible to study antigen induced activation at early time point. Hence proliferation was studied by stimulating normal splenic T cells with polyclonal mitogen Con A and superantigen SEB, using APC's of normal mice and those with

chronic GVHD, and cultures were pulsed at different time points 48h, 72h and 120h.

The response observed at 48h in T cell cultures, with APC's of both normal F1 mice and those with chronic GVHD, showed normal proliferation. Thereafter the proliferative response declined, possibly due to apoptosis. The decline in proliferation was steeper in cultures with APC's of mice with chronic GVHD.

Data suggest that observed antigen specific nonresponsiveness induced by repeated immunization with *M.vaccae* in mice with chronic GVHD, may also be due to abnormal antigen presentation resulting in to clonal loss.

Based on these findings it can be proposed that in chronic GVHD the parental T cells recognizing other parental allo-MHC, secrete large amounts of cytokines. These cytokines can induce changes in the class II expressing APC's i.e. dendritic cells, macrophages, B cells, by upregulating the costimulatory molecules, thus altering the antigen presentation parameters leading to greater apoptosis of activated T cell clones. Equilibrium between cell death and proliferation resulting from antigenic stimulation is largely responsible for maintaining the size of and phenotypic balance of the mature peripheral T cell pool. Russell (339) has pointed to the APC as a principal regulator of the two opposing responses namely proliferation and death due to apoptosis. He proposed that lineage and anatomical location of the APC plays an important role in determining whether a T cell responding to presented antigen remains capable of further proliferation or dies. He has proposed a higher rate of apoptosis of antigen activated clones in lesions in tissues as compared to the nodes (339). The cytokines of activated T

cells in lesions can induce a greater degree of apoptosis. Evidence of induction of tolerance (i.e. loss of activated clones) due to inflammation in tissues reported by Fields and Loh (444) supports this view. Similar changes induced in APC's of lymphoid tissues in chronic GVHD can lead to higher rates of apoptosis when T cell clones are activated in nodes. This can explain our observation of repeated immunization causing a pathological clonal loss in chronic GVHD, leading to unresponsiveness to the antigen. One can expect in chronic GVHD, a similar clonal loss to occur, due to natural exposure to the environmental antigens, idiotypes and self antigen cross reactive with foreign antigen. Further, such a clonal loss, occurring over long periods, can cause the observed non responsiveness of Th cells to foreign antigens (which is an oligoclonal response), without severely depleting the response to Con A or allo-MHC, because these are polyclonal responses.

The changes brought about in APC's in chronic GVHD, can have paradoxical effects. Animals can be made responsive to antigens which are normally ignored by T cells. This is evident in chronic GVHD, wherein the affected animals/patients produce antibodies to variety of self antigens i.e. erythrocytes, DNA. In direct contradiction to the observation of Fields and Loh (444) reported earlier, some form of tissue inflammation (and consequent changes in APC's) has been reported to be essential for inducing autoimmune disease (518), even when the precursor frequency of T cells recognizing a given autoantigen is high.

Thus in chronic GVHD, autoimmune disorders seen can be one side of the coin, nonresponsiveness being the other side.

Bone marrow derived stromal cells of thymus also behave as APC's and they serve the important function of affinity based negative selection of clones recognizing the antigens presented by them (101,401,396). Chronic GVHD, which can induce changes in the APC's in the peripheral lymphoid tissues, can also bring about similar changes in the thymic stromal APC's (519,520). This can alter the presentation parameters in the thymus , consequently leading to excessive negative selection. Thus emergence of nonresponsiveness could also be due to thymic stromal cells being affected. Hence the presentation efficiency of thymic cells was also investigated in these mice with chronic GVHD. Thymic cells in general were found to be inferior for presentation of antigen when compared with spleen cells. The antigen specific proliferative response induced by thymic APC's of animals with chronic GVHD was quite poor in comparison with that induced by thymic APC's of normal mice. This finding is significant, even though it does not imply excessive negative selection in the thymus.

A significant point in this context is the similarity in the composition of T cells in the peripheral lymphoid compartment of adult thymectomised mice and in chronic GVHD. In normal mice, naive T cells (expressing CD45 high molecular weight isoform) outnumber the memory/activated T cells (CD45RO+) by a factor of 2 (219). In adult thymectomised mice (219) and in chronic GVHD patients (521,522) the CD45RO+ T cells outnumber the former. Cytokines secretion by Con A stimulated spleen cells of adult thymectomised mice also reflects the changes in the relative frequencies of naive and memory/activated T cells. Normal spleen cells produce only IL-2 (227,228), whereas the spleen cells of adult thymectomised mice produce higher levels of IL-2 and the other cytokines such as IL-4 and IL-10 on stimulation with Con A (219). In

the light of this finding, the cytokine secretion of spleen cells of mice with chronic GVHD of 3 months duration was investigated. Indeed, not only higher levels of IL-2 were produced, but other cytokines IL-4, IL-10 were also generated. Thus it was concluded that composition of the peripheral T cells of mice resembles that of adult thymectomised mice. In view of this finding, abnormal antigen presentation by the thymic APC's of mice with chronic GVHD acquires greater significance. Possibility that the bone marrow derived APC's in thymus, like their counterparts in the peripheral lymphoid tissues are also altered, leading to greater negative selection seems very likely. Thus T cells of mice with chronic GVHD appear to be deleted over a period, both by central and peripheral mechanisms in which abnormal APC's seem to play an important role.

Appearance of activated/memory profile of T cells in the peripheral compartment, due primarily to polyclonal activation occurring as a result of changes in the APC's, has another important implication. Activation of T cells results into expression of both Fas and FasL. Therefore presence of a large number of activated T cells expressing FasL even as bystander cells might provide apoptotic signal to the antigen activated T cells. Further activated parental T cells, capable of recognizing the MHC class II alloantigen on the F1 APC's, can form a cluster with antigen bearing APC, and the responding F1 T cells. Since the parental T cells are activated, they would express FasL and provide the apoptotic signal to the antigen responding F1 T cells. It is known that activation of mature T cells causes downregulation of Bcl-2 in parallel with upregulation of Fas (215,346). The Fas antigen is one of the target molecules that is triggered during T-T interactions, causing death upon antigen specific recognition by an effector cell that bears a Fas L (345,346).

Bcl-2 expression is most prominent in CD45RB^{hi} (naive) T cells which express low levels of Fas antigen. CD45RO⁺ (activated/memory) T cells, by contrast, are strongly positive for Fas but express only low levels of Bcl-2 (215) . This reciprocal pattern of Bcl-2 and Fas expression as well as the delay in sensitivity to Fas mediated death of freshly activated cells (437,438), suggest that activation-induced death of mature T cells applies preferentially to previously activated T cells. This suggests that chronic activation brought about in GVHD is responsible for the AICD resulting into progressive clonal loss.

II - Activation of CD8+ Ts cells facilitated by superantigen interaction.

It is evident from our studies that endogenous or exogenous infectious MMTV play a significant role in the activation of CD8+ Ts cells by class II presented antigens.

Our earlier studies on i.p. immunization with *M.vaccæ* (4,41), had demonstrated expansion of CD8+ Ts cells that are capable of competing for growth factor, IL-2 and thus suppressing the immune response of simultaneously expanded CD4+ Th cells. These activated CD8+ Ts cells were shown to recognize the 65-kDa antigen of *M.vaccæ* presented by MHC class II. Several other investigations have reported such similar aberrant MHC class II restriction of the suppressor T cells (462).

The reasons already described in the review (see pg72) and results (see pg113), prompted us to investigate the role of superantigens in the expansion of CD8+ Ts cells by the 65-kDa antigen. This was undertaken to answer the main debated issue of suppression, namely class II restriction of suppressor cells of CD8 phenotype. This issue was studied using four different strains of mice, C3H, B6 and two sets of F1 hybrids of the above two strains, which gave us different combinations of IE and MMTV derived superantigen.

Immunization of all the four strains with *M.vaccæ* by the i.d. route generated Th responses specific for the immunodominant 65-kDa antigen of the organism. This conclusion was based on the observations that the i.d. immunized mice of all the four strains

showed DTH, lymphoproliferative and antibody responses against the 65-kDa antigen. The responses of the i.p. immunized mice were not uniform. The C3H and (C3H x B6)F1 mice failed to mount the DTH and the lymphoproliferative responses on i.p. immunization. This was observed to be due to expansion of CD8⁺ Ts cells following i.p. immunization only. The i.p. primed CD8⁺ T cells were able to suppress, on adoptive transfer to the syngeneic hosts, all the three responses, i.e. DTH, lymphoproliferative and antibody responses, generated on i.d. immunization of the recipients. Similarly the i.p. primed CD8⁺ T cells when mixed with i.d. primed spleen cells, abrogated the in-vitro proliferation of the latter induced by the 65-kDa antigen. Therefore it was concluded that the phenomenon could be considered as "suppression" and the cells causing it as the "suppressors". Recognition of the antigen by the CD8⁺ Ts cells appeared to be IE restricted. However, based on the evidence of inhibition by anti-IA and anti-IE antibodies the question of restriction versus dependence could not be unequivocally resolved.

In contrast to the C3H and (C3H x B6)F1 mice, the B6 and (B6 x C3H)F1 mice did mount both the DTH and lymphoproliferative responses to the i.p. immunization with *M.vaccæ*. Adoptive syngeneic transfer of i.p. primed splenic CD8⁺ T cells of these mice failed to abrogate the DTH and lymphoproliferative responses to i.d. immunization of the recipients. These results appeared to suggest that the phenomenon of generation of antigen specific suppression by i.p. immunization was not universal.

Since the CD8⁺Ts were earlier shown to be IE restricted /dependent, the inability of B6 mice to expand the suppressors on i.p. immunization was thought to be due to lack of IE expression.

However this explanation could not be extended to (B6 x C3H)F1 mice because they would express IE of C3H parent.

The observations of adoptive transfer experiments of i.p. primed cells of the F1 strains into each other and into the parental strains brought out some interesting findings that seem to offer explanations as to why no suppression was observed on i.p. immunization of B6 and (B6 x C3H) F1 mice. Adoptive transfer of i.p. primed CD8 enriched cells of (C3H X B6)F1 mice abrogated the response of (B6 x C3H) F1 mice to i.d. immunization. Most surprising were the results of the experiments with i.p. primed CD8 enriched cells of (B6 x C3H)F1 mice. Even though the cells, on adoptive transfer, could not abrogate the responses of the syngeneic (B6 x C3H) F1 or parental B6 strains, to i.d. immunization, they could successfully suppress the responses of parental C3H or the (C3H x B6)F1 strain to i.d. immunization. Both DTH and lymphoproliferative responses were suppressed. Thus it appeared that for effective abrogation of *in vivo* response to i.d. immunization, the CD8+Ts cells of i.p. primed F1 mice, either had to come from (C3H x B6) F1 or had to be transferred into C3H or (C3H x B6)F1. Some factor available in C3H or (C3H x B6) F1 mice seemed to be essential for the CD8+T suppressors to be effective. This indicated the possibility that i.p. priming of (B6 x C3H) F1 mice did expand CD8+Ts cell clones but not to the extent required to suppress the responses of simultaneously primed CD4+Th cells; and therefore no suppression was observed in them. Further, the putative factor available in the C3H or the (C3H x B6) F1 strains enabled to expand adequately when transferred into these strains so as to abrogate their responses to i.d. immunization; the factor being unavailable in (B6 x C3H)F1. In order to investigate this possibility, irradiated non immune normal C3H or (C3H x B6)F1 cells were transferred into

(B6 × C3H)F1 mice, who were then given i.p. immunization with *M.vaccae*. Indeed, such a transfer enabled the (B6 × C3H)F1 mice to suppress their response to i.p. immunization, indicating that the putative factor transferred along with irradiated cells of C3H and (C3H × B6)F1 cells respectively, in fact enabled the recipients to generate suppression on i.p. immunization. The putative factor could be the MMTV derived superantigen because MMTV infection is present only in C3H and (C3H × B6) F1 mice.

Some of the significant observations reported earlier and repeated by us, on CD8⁺ Ts cells in i.p. primed mice of the same strains, also point to the role of superantigen in their activation (46). The recognition of the epitope/s of the 65-kDa antigen by the CD8⁺ Ts cells appeared to be quite promiscuous, whenever the APCs expressed both IA and IE, IE was the presenting element preferred by the CD8⁺Ts cells for recognition. However, when the same population of primed CD8⁺Ts cells were presented the 65-kDa antigen by APCs expressing only IA, the recognition did take place. Most significant observation was that the primed CD8⁺ Ts cells recognized the 65-kDa antigen even on allogeneic IE. These findings indicate that the recognition of the 65-kDa antigen by the CD8⁺ Ts cells was class II, IE, dependent and not restricted. These are the properties of superantigen induced activation (58,492,494). Therefore, the possibility that 65-kDa itself behaved like a superantigen, was investigated and ruled out.

It is significant that i.d. priming did not stimulate “suppressors”. It has been reported that the *mtv* (integrated MMTV genomes) encoded endogenous superantigens are not expressed by the dendritic cells (495). These cells constitute the APC's in the dermis. It is likely that even MMTV superantigen is not expressed by

these cells. Therefore, one can expect i.d. immunization not to generate Ts response.

In the light of these arguments, a role for MMTV superantigen of CD8+Ts cells was considered very likely. Blockade of 65-kDa induced CD8+Ts cell activation by the anti-IE antibody described in several experiments could be due to blockade of interaction between the superantigen and the IE on the APC. ORF protein of MMTV which is a superantigen, interacts with V β 14. Therefore, blockade of CD8+ Ts cell activation with anti-V β 14 antibody was also attempted. The antibody failed to block suppression. Anti-TCR antibodies are known to stimulate T cells by virtue of their ability to modulate the receptor. It is therefore likely that the anti-V β 14 antibody did not block suppression because it is mitogenic and activates the very cells whose interaction with the superantigen the antibody is supposed to block. Therefore, the ability of the antiV β 14 antibody to stimulate naive C3H spleen cells to proliferate, was investigated and indeed the antibody was found to be mitogenic.

Since i.d. immunization did not activate the CD8+Ts cells it was decided to investigate, if *in vitro* modulation of V β 14+TCR by the antibody in conjunction with the 65-kDa antigen would activate the antigen specific CD8+Ts cells in i.d. primed spleen cell suspensions. Indeed, the antibody appeared to suppress *M.vaccae* specific *in vitro* proliferation of the i.d. primed spleen cells and had no effect on the proliferation induced by Con A. In order to ascribe the explanation of this effect to the activation of CD8+ Ts cells due to TCR modulation by anti V β 14 antibody, it was essential to rule out the possibility that anti-V β 14 antibody did not block recognition of 65-kDa antigen by CD4+ Th cells. It is important to note that the proliferative response of the i.p. primed cells, though to that of i.d.

primed cells, is also due to CD4⁺ Th cells. This response could be blocked by anti-V β 14 antibody (Table II.10) implying that the antibody did not block recognition of 65-kDa antigen by CD4⁺ Th cells. However, based on this data it was not certain that the anti-V β 14 antibody suppressed the proliferative response by modulating the TCR of CD8⁺Ts cells. Also we could not be unequivocally certain of the specificity of suppression against 65-kDa antigen. It is significant for *in vitro* suppression of 65-kDa specific proliferation of i.d. primed Th cells, anti-V β 14 antibody (which was mitogenic to naive cells) was required. In contrast for activation of CD8⁺Ts cells in the cultures of i.p. primed spleen cells 65-kDa antigen alone was adequate. In order to be certain that the CD8⁺ Ts cells in the spleen cell suspension of i.d. primed mice, can be activated by 65-kDa antigen and anti-V β 14 antibody together, we need an *in vitro* assay of CD8⁺ Ts cell activation. Such an assay, developed by us earlier (46), is presented in section 4. The same assay was employed by us to investigate if CD8⁺ Ts cells in the spleen cell suspension of i.d. primed mice can be activated by 65-kDa antigen and anti-V β 14 antibody together. Indeed our data confirm that for activation of CD8⁺ Ts cells in the i.d. primed spleen cell suspension, both 65-kDa antigen and anti-V β 14 antibody were required. In contrast for their activation in the spleen cell suspension of i.p. primed cells 65-kDa antigen alone was adequate. It thus emerges that some factor available during i.p. priming, enables the V β 14 positive CD8⁺ Ts cells to expand and suppress 65-kDa specific CD4⁺ Th cell response.

Therefore, it is speculated that 65-kDa specific CD8⁺Ts cells expanded in C3H and (C3H $\times$ B6)F1 mice by i.p. immunization with *M.vaccæ* are most likely V β 14⁺T cells and the MMTV superantigen which has the same V β specificity is involved in their activation by the 65-kDa antigen. It is also speculated that the MMTV superantigen

is not available for activation of the CD8+Ts cells when immunization is done by the i.d. route, because the local APCs, namely the dendritic cells do not express the superantigen for reasons elaborated earlier.

Salgame *et al* (462) have reported similar experimental results using anti-V β 8 antibodies, and human CD8+ Ts cell clones isolated from the lepromatous leprosy patients. The Ts clones could inhibit antigen specific proliferation of peripheral blood lymphocytes of the lepromatous patients. Their studies indicate that addition of anti-V β 8 antibody could block the suppression.

The role of MMTV superantigen in the activation of CD8+Ts cells by a class II presented bacterial antigen can be explained by the following hypothesis originally proposed by Janeway (496,497). A processed peptide antigen and a non processed superantigen interact with MHC class II and TCR at different sites. As a consequence, theoretically, a superantigen interaction can enhance the peptide-MHC interaction with TCR. Peptide-MHC complexes with low affinity for TCR (such as class II processed antigens for TCR of CD8+T cells) which are normally ignored by T cells, can be rendered immunogenic by such enhancing effect of a superantigen. For this to occur effectively, the superantigen ought to be weak, otherwise the relevant T cell clones will be deleted by central or peripheral mechanisms involved in deletion of superantigen recognizing clones (425).

Therefore strong endogenous *mtv* coded superantigens would not be able to participate in such an interaction. Incidentally, the MMTV (C3H) superantigen has been reported to be a weak superantigen not capable of exhaustive deletion (525) . The putative

suppressors CD8⁺T cells in our model have been shown to recognize 65-kDa epitopes on MHC class II . Because of aberrant MHC, the required affinity for activation of CD8⁺T cells would not be achieved. The weak ORF superantigen of MMTV can provide the additional interaction for increasing the affinity, by a bridge on the lateral surfaces of V β domain of TCR and invariant region of IE. Thus, the hypothesis also offers a reasonable explanation for the alleged class II restricted recognition of antigen by the CD8⁺Ts cells.

The phenomenon of suppressor to i.p. immunization with *M.vaccae* is not restricted to these strains having chronic MMTV infection. In fact, several other IE positive strains, studied by us earlier mounted a suppressive response on i.p. immunization (46,489). Therefore, it was decided to investigate, if MMTV derived *mtv* coded endogenous superantigens played a similar role. No correlation between expression of *mtv* coded superantigens and generation of immune suppression by i.p. immunization with *M.vaccae*, was observed. This could be because most strong *mtv* coded superantigens would delete the T cells capable of interacting with them. In Swiss white mice, the effect could be due to exogenous MMTV (SW) infection. In BALB/C3H also such a superantigen has been described, due to contamination of the strain with MMTV (SW) (524). However, prevalence of such an exogenous superantigen due to any known MMTV or other infection has not been described in all the strains. Further the suppression seen in (B6 $\times$ BALB)F1 mice is difficult to explain, because male BALB/C3H can not transmit MMTV.

Data on (B6 $\times$ C3H)F1 mice also indicate that even in the absence of the superantigen mediated interactions, some expansion of CD8⁺Ts cells does occur . However, the size of the expanded

"suppressor" pool appeared to be inadequate to abrogate the *in vivo* responses.

It is possible that in all these situations, as yet unrecognized one or more endogenous or exogenous weak superantigens may be playing the crucial role in CD8⁺ T cell activation. These superantigens could originate in the acquired viral genomes such as *mtv* or even cellular genes as proposed by Janeway (497,528).



SUMMARY AND CONCLUSIONS

Though the essential function of the immune system is to react against foreign antigens and differentiate into effectors, sometimes antigen induced activation results into nonresponsiveness. By employing two different models of activation induced nonresponsiveness we decided to investigate the mechanisms induced by immunization. Our study was done in two parts. Experimental model of chronic GVHD was used in the first part of the study, where characteristic abnormalities related to T cell activation are observed. The role of APCs in the induction of nonresponsiveness was studied in this model. Second model used, was our earlier published model of induction of nonresponsiveness on i.p. immunization due to activation of CD8+Ts clones capable of competing with CD4+Th cell clones for IL-2. The role of MMTV superantigen in the activation of competing CD8+Ts clones was studied in the second part of the study.

Chronic GVHD was induced by injecting parental C3H spleen cells into (B6 x C3H)F1 mice. The recipients when tested after 3 months of cell transfer, showed marked splenomegaly indicated by GVH index of greater than 1.34 and also showed evidence of autoantibodies against red cells and dsDNA indicating establishment of chronic GVHD, these animals showed T cell nonresponsiveness to a randomly selected immunogen, *M.vaccinae* vaccine. The animals were unable to mount both DTH and a proliferative responses. However, the response to polyclonal mitogen Con A was relatively unaffected. The immune defect in mounting a Th response towards *M.vaccinae* was not observed in animals with chronic GVHD of only 3 weeks duration. However, with 3 repeated immunizations of these mice at one week intervals, the Th response to *M.vaccinae* were progressively declined. All the three responses

tested, namely DTH, lymphoproliferative and the antibody response against 65-kDa antigen of *M.vaccae* were adversely affected. In normal F1 mice such nonresponsiveness was not induced after 3 repeated immunizations. The nonresponsiveness observed was specific towards *M.vaccae*, as the response to immunization with *Salmonella enteritidis*, after two immunizations with *M.vaccae* was normal. Pulse treatment with Cyclosporin A, 0.5mg/mouse, given on 5 consecutive days along with every immunization of *M.vaccae* prevented the emergence of nonresponsiveness after 3 repeated immunizations. Based on these results it was concluded that repeated activation of T cells of mice with chronic GVHD induces nonresponsiveness, probably as a result of loss due to AICD.

A known model of AICD namely induction of nonresponsiveness by in-vivo treatment with superantigen, was then employed to investigate if in chronic GVHD there is excessive AICD. Administration of a single dose of SEB (25µg/mouse), induced loss of SEB responding clones in both normal F1 and those having chronic GVHD, however, the extent of loss was greater in mice with chronic GVHD, confirming that in chronic GVHD AICD, was excessive.

Abnormal antigen presentation can be one of the reasons for the excessive AICD. Hence antigen presentation by APCs of normal F1 mice and those with chronic GVHD were studied. i.d. primed splenic T cells were stimulated *in-vitro* with *M.vaccae* antigen presented by irradiated naïve spleen cells of normal F1 and those with chronic GVHD in a standard 6 day lymphoproliferative assay. Indeed the response generated by chronic GVHD was found to be poor. As peak proliferation of activated T cells occurs at 24-48h, single time point observation of proliferation at 120h was not adequate to conclude that the poor response was infact due to

excessive AICD. Hence proliferation at different time points of normal naïve T cells induced by, polyclonal mitogen ConA and a superantigen SEB, in presence of irradiated spleen cells of normal F1 or those with chronic GVHD, was investigated. The 48h proliferative responses with both the sources of APCs were comparable. As expected the proliferation declined subsequently due to AICD. However the decline in proliferation was rapid and steeper in cultures with APCs of mice with chronic GVHD. It was therefore concluded that APCs of mice with chronic GVHD were inducing greater AICD.

Based on these findings, emergence of antigen specific nonresponsiveness by repeated immunizations of mice with chronic GVHD is ascribed to abnormal antigen presentation by APCs, causing excessive AICD. Pathology of chronic GVHD involves activation of parental T cells by alloantigens of the other parent on F1 cells, which results in cytokine production and consequent upregulation of B7 and other adhesion markers on APCs. AICD induced in the context of proper costimulation, is usually due to repeated ligation of TCR and is usually augmented by cytokine such as IL-2. Considering the possibility that the costimulatory ligands on the APCs of mice with chronic GVHD are likely to be upregulated, enhanced AICD in chronic GVHD could be because of greater production of cytokines by activated T cells. Emergence of nonresponsiveness to a foreign antigen observed in the natural course of chronic GVHD could also be due to a similar T cell loss on natural exposure to the environmental antigens, idiotypes and self antigens cross reactive with foreign antigen. The upregulated costimulatory molecules of the APCs in chronic GVHD, may also render self antigens normally ignored, immunogenic, leading to autoimmunity. Requirement of upregulation of costimulatory molecules by a local

inflammatory process, for induction of autoimmune disorders has been reported.

Similar to excessive AICD observed in periphery, one can not rule out excessive negative selection in the thymus due to changes in the stromal APCs as a result of chronic GVHD. APCs of bone marrow derived thymic stromal cells also express MHC class II and present antigens and hence similar changes can be expected. Antigen presentation by irradiated thymus cells of mice with chronic GVHD as APCs, was indeed found to be poor.

Naïve peripheral T cells show preponderance of cells with high molecular weight isoform of CD45, at a ratio of 3:1. It has been reported that the composition of peripheral T cells is reversed in chronic GVHD. The naïve T cells secrete only IL-2 when stimulated with ConA. In contrast CD45RO⁺ cells secrete other cytokines in addition to IL-2, pattern characteristic of activated/memory cells. Similar cytokine secretion pattern was observed in mice with chronic GVHD. It may be noted that similar changes in T cell composition occurs in adult thymectomised mice.

Thus emergence of nonresponsiveness to foreign antigen in chronic GVHD could be due to changes in peripheral as well as thymic APCs causing excessive AICD in the periphery and excessive negative selection in the thymus.

In second part of the study, the reason for class II restriction /dependence in activation of CD8⁺Ts cell clones capable of competing for IL-2 was investigated.

Earlier we have shown that i.p. immunization with *M.vaccæ* results into simultaneous activation of CD4+Th and CD8+Ts cells. CD8+Ts cells were further shown to aberrantly recognise the antigen on MHC class II and compete for growth factor IL-2 and thus suppressing the response of CD4+Th cells. Reasons for aberrant recognition of antigen on MHC class II despite being CD8 phenotype was investigated in this study.

Our earlier reported work on MHC restriction requirement showed that the expanded clones of CD8+T cells, could recognise antigen on MHC class II - IA or IE or even on allogeneic IE, suggesting that the recognition was in fact IE dependent and not restricted. Since i.d. immunization did not expand CD8+Ts cells, their expansion by i.p. route suggests the possibility that some factor was being provided which is MHC II-IE dependent and not restricted. Such a factor can be a superantigen of MMTV, because it is IE dependent for activation and also mice are known to be infected with MMTV. Further it is known that endogenous *mtv* derived superantigens are not expressed by dendritic cells and macrophages but are expressed by B cells. A similar situation could also be operating for infectious MMTV. Since peritoneum contains B cells, the superantigen would be available for activation when immunization is done by the i.p. route. In order to ascertain the possibility of a role for MMTV superantigen in CD8+Ts activation by mycobacterial 65-kDa antigen, four strains were selected to give different combinations of IE expression and superantigens.

C3H mice are IE positive and are chronic carriers of infectious MMTV, B6 strain is IE negative and does not carry MMTV infection. Since MMTV is transmitted only maternally the two sets of F1 raised from the above mice gave two different combinations of expression of IE and the superantigen. (C3H female x B6 male)F1, like C3H

mothers are MMTV carriers and are IE positive whereas (B6 female x C3H male)F1 express IE but do not carry MMTV infection. All these four strains were immunized and the generation of suppressive response investigated them.

i.d. immunization of all the four strains with *M.vaccae* generated DTH and lymphoproliferative responses against the sonicate/65-kDa antigen of the organism. Responses to i.p. immunization depended upon the C3H and (C3H x B6)F1, both IE positive and MMTV carriers, showed antigen specific immune suppression mediated by CD8⁺Ts cells. B6 and (B6 x C3H)F1 mice, the former IE and MMTV negative and the latter IE positive but MMTV negative mounted both DTH and lymphoproliferative responses on i.p. immunization. Adoptive transfer experiments of i.p. primed cells of the F1 strains into each other and into the parental strains showed some curious findings. i.p. primed (C3H x B6)F1 cells abrogated the responses of (B6 x C3H)F1 mice to i.d. immunization. In contrast (B6 x C3H)F1 i.p. primed cells on adoptive transfer could not abrogate the response of the syngeneic or parental B6 mice to i.d. immunization, but could successfully suppress the responses of parental C3H or (C3H x B6)F1 strain to i.d. immunization. Thus it appeared that some factor available in C3H or (C3H x B6)F1 mice seemed to be essential for the CD8⁺T suppressors to be effective. This was confirmed by transfer of irradiated naïve spleen cells of normal C3H or (C3H x B6)F1 mice into (B6 x C3H)F1 mice prior to immunization. Such a transfer enabled the recipients to generate specific immune suppression on i.p. immunization and it had no effect on i.d. immunization.

The fact that dermal dendritic cells do not express superantigen and i.d. immunization does not generate suppression, further emphasized the role of superantigen present in the MMTV

carrier strains to be responsible for the expansion of CD8⁺T suppressors. It was speculated that the superantigen interacting with external domains of MHC class II (IE) of the APCs and the V β specificity on the external domains of the TCR, may enhance the affinity of recognition of class II presented 65-kDa antigen of *M.vaccinae* by the CD8⁺T cells. Since ORF protein of MMTV (C3H) superantigen interacts with V β 14 TCR, blockade of CD8 activation by anti-V β 14 was attempted. Anti-IE could block the suppression but anti-V β 14 antibody failed to block the suppression. The anti-TCR antibodies were in fact found to be mitogenic to naïve T cells. Since anti-V β 14 antibodies could modulate the TCR, it is logical to expect them to modulate the receptor of CD8⁺T cells also and possibly enhance the activation of specific clones by 65-kDa antigen of *M.vaccinae*. In our model, i.d. immunization had never lead to activation and expansion of CD8⁺Ts cells . Which we believed to be because the superantigen was not available on the dermal dendritic cells. Since anti-V β 14 could modulate the TCRs, it was decided to investigate if 65-kDa antigen induced proliferation of i.d. primed could be suppressed by anti-V β 14 antibody which was mitogenic to naïve T cells. Indeed the antibody suppressed the antigen specific proliferation but had no effect on ConA induced proliferation. However, in this observation we could not unequivocally certain that anti-V β 14 antibody achieved this by activation of 65-kDa specific CD8⁺T cell clones by acting in concert with the antigen. It was essential to have antigen specific CD8⁺Ts cell activation assay. Such an assay described earlier in our laboratory was employed. In this assay, spleen cells of i.p./i.d primed or non primed were stimulated with exogenously added IL-2. All three types of cells proliferated in response to IL-2. Addition of antigen to non primed cells made no difference. Addition of antigen to i.d. primed cells lead to enhancement in proliferation. In contrast addition of antigen to i.p.

primed cells lead to suppression of proliferation. The proliferating cells were CD4⁺T cells, not necessarily specific for antigen. The suppression would be abrogated by removal of CD8⁺T cells by antibody and complement treatment. It has further been shown earlier that the i.p. primed CD8⁺T cells could absorb IL-2 and the absorptive capacity was enhanced by antigen. Thus this was truly an assay of T suppressor activation. The same assay was employed. I.d. primed spleen cells were stimulated with IL-2. Addition of 65-kDa antigen lead to enhancement in proliferation, as seen earlier. Addition of anti-V β 14 antibody had no effect. Addition of anti-V β 14 and antigen lead to suppression of proliferation. This data clearly indicated that the anti-V β 14 antibody conjunction with 65-kDa antigen induced activation of CD8⁺Ts cells in the spleen cell suspension of i.d. primed mice. In contrast, in the suppression of i.p. primed mice, antigen alone was adequate. Based on this data it was concluded that the additional ligand, naturally available in the peritoneum for the activation of CD8⁺Ts cells by the mycobacterial 65-kDa antigen, had the same specificity, i.e. V β 14. It was therefore concluded that the peritoneum, provided the additional ligand required for activation of CD8⁺Ts cells by aberrant MHC class II, of IE specificity.

All inbred and outbred strains of mice express some endogenous superantigen/s coded by *mtv* genome. Hence correlation of endogenous superantigen and generation of suppression by i.p. immunization was studied using different strains. No correlation between an endogenous coded superantigen and suppressor response to i.p. immunization was observed. Strong endogenous superantigens cause deletion of the reactivator clones, thus explaining lack of correlation between the suppressor response and endogenous superantigen expression. MMTV of C3H is known to be a weak superantigen which can not cause exhaustive deletion. Hence a role for weak endogenous superantigens and the so called "cellular

superantigens" (as suggested by Janeway) in activation of CD8⁺Ts cells by the mycobacterial 65-kDa antigen appears likely.

Thus two different models of induction of antigen specific nonresponsiveness as a consequence of antigenic stimulation, were investigated, to analyse different mechanisms for emergence of non responsiveness.

APPENDIX

Appendix I

Preparation of Media, Reagents and Buffers

I.a)Doub and Youman's medium

Aspargine	5.0gm.
KH ₂ PO ₄	5.0gm.
K ₂ SO ₄	0.5gm.
Glycerol	20ml.
Distilled water	1000ml.

All the ingredients were dissolved in distilled water. After thorough mixing the pH was adjusted to 6.8 to 7 by adding appropriate amount of 1N NaOH and

Magnesium citrate	1.5gm.
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The solution was mixed properly and heated if necessary to dissolve magnesium citrate and then dispensed into tubes or flasks. Medium was sterilized by autoclaving at 15 lbs and 121°C for 20min.

I.b)Liquid Broth

Bacto-tryptone	10.0gm.
Yeast Extract	5.0gm.
Sodium Chloride	5.0gm.
Glucose	1.0gm.
Distilled Water	1000ml.

The ingredients were mixed and dissolved by keeping in boiling water bath. After cooling, the pH was adjusted to 7.0. The medium was filtered and sterilized by autoclaving at 110°C for 10min. at 10lb pressure.

I.c) Phosphate buffered saline (PBS) (pH 7.2, 0.15M)

Sodium chloride	8.0 g
Di-potassium hydrogen phosphate (K_2HPO_4)	1.21 g
Potassium di-hydrogen phosphate (KH_2PO_4)	0.34 g
Distilled water	1000 ml

I.d) Tris buffer (pH 7.8, 0.01M)

Tris	1.21gm.
Distilled water	1000ml.

Tris was dissolved in distilled water and pH was adjusted to 7.8 by 0.1NHcl.

For the Lysozyme digestion 0.292gm (0.01M) of EDTA was added in 0.01M Tris buffer.

I.e) Tris glycine (0.025 M Tris, 0.192 M ,glycine pH8.3)

Tris	3.03g
Glycine	4.4 g
Distilled water	1000 ml

I.f) Carbonate-bicarbonate buffer (0.05M, pH 9.6)

Na₂CO₃ ———0.53 gm. in 100ml distilled water (0.05M)

NaHCO₃———0.42gm in 100ml distilled water (0.05M)

Approximately 10ml of Na₂CO₃ (0.05M) was added to 90ml of NaHCO₃ (0.05M) to prepare 0.05M Carbonate buffer with pH 9.6.

I.g)Scintillation fluid

1-4 bis (5-phenyl oxazolyl) benzene (POPOP) 0.3016g

2-5 diphenyl oxazole (PPO) 3.968 g

Toulene (GR) 1000 ml

PPO and POPOP were dissolved in 800ml toluene in a volumetric flask mixed thoroughly. After complete dissolution the volume was made to 1.0 liter with toluene.

I.h)Citrate buffer (0.15M, pH 6)- (Substrate buffer)

Na-Citrate ———0.24gm in 100ml Distilled water (0.15M)

Citric acid ———0.21gm in 100ml Distilled water (0.15M)

Approximately 5 ml. of citric acid (0.15M) was added in 45ml. of Na-Citrate (0.15M) to prepare the 0.15M Citrate buffer with pH to 6.

I.i) Phosphate buffered saline -Tween (PBS-T)

Phosphate buffered saline (0.15M, pH7.2) 1000 ml

Tween -20 0.5 ml.

I.j) Saline Tween

Sodium chloride NaCl 8.5 g

Distilled water 1000 ml

After dissolving NaCl, Tween 20 added 0.5 ml.

I.k)1mM EDTA PBS

EDTA 0.37gm. Added in 1000 ml. PBS. (0.15M)

Appendix II

Procedures

II.a) Estimation of protein by Lowry's method

Reagents :

Reagent A-1

Sodium potassium tartarate	0.2gm.
Distilled water	10ml.

Reagent A-2

Cupric sulphate	0.1gm.
Distilled water	10ml.

Reagent A-3

Sodium Carbonate	2.0gm.
Sodium hydroxide (0.1N)	0.4gm.
Distilled water	100ml.

Reagent A

1ml. A-1 + 1ml. A-2 was mixed and added to 100ml. of A-3.

Reagent B

Folin-ciocalteu's reagent (Sisco) was diluted 1:1 with distilled water just before use.

Standard protein solution.

10mg of Bovine serum albumin (BSA Sigma) was dissolved in 5ml. distilled water. This was further dissolved 1:10 with distilled water to get 200 μ g/ml of protein solution which was used as a standard for the estimation of protein content.

Assay procedure

A set of clean glass tubes was taken to which 0.1, 0.2, 0.3, 0.4 and 0.5 ml. of standard BSA was added in duplicate. The volume of each tube was made upto 0.5ml. with distilled water. Blanks of 0.5ml. distilled water were also set up. The test solutions also were taken in 0.5ml. quantity with appropriate dilutions for the protein estimations.

2.5ml. reagent A was added to each tube. The contents of each tube were mixed and the tubes were allowed to stand at room temperature for 15min.

0.25ml. of reagent B was added to each tube with vigorous shaking and the tubes were allowed to stand at room temperature for 30min.

Optical density (OD) readings were taken at 660nm. Adjusted against the distilled water blank. A graph of OD against protein concentration was plotted from which the protein concentration of test sample was calculated.

II.b) Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

This was done employing the method of Laemmli (526).

Gels for SDS-PAGE

<u>Reagents</u>	<u>Resolving gel</u>	<u>Stacking gel</u>
Acrylamide stock solution	5.0ml.	0.8ml
Distilled water	6.8ml	3.6ml
Resolving gel buffer	3.0ml	-
Stacking gel buffer	-	0.5ml
10%SDS	150 μ l	50 μ l
TEMED	7.5 μ l	5 μ l
10% Ammonium persulphate (prepared fresh)	50 μ l	17 μ l

Reagents

a) Acrylamide stock solution

Acrylamide	30gm.
Bisacrylamide	0.8gm.
Distilled water	100ml.

The two ingredients were dissolved in 50ml distilled water and the final volume was adjusted to 100ml.

b) Resolving gel buffer (1.875M Tris-HCl,pH8.8)

Tris	27gm.
Distilled water	100ml.

Tris was dissolved in 60ml. distilled water. The pH was adjusted to 8.8 with 5N HCl. The volume was then made up to 100ml.

c) Stacking gel buffer (1.25M Tris-HCl, pH 6.8)

Tris	15.12gm.
Distilled water	100ml.

Tris was dissolved in 60ml. distilled water. The pH was adjusted to 6.8 with 5N HCl. The volume was then made up to 100ml.

d) Reservoir buffer (0.025M Tris, 0.192M glycine, pH 8.3)

Tris	3.03gm.
Glycine	14.4gm.
SDS	1gm.
Distilled water	1000ml.

e) Sample buffer (2x)

1.25M Tris-HCl, pH6.8 (stacking gel buffer)	5ml.
SDS	2gm.
2-mercaptoethanol	5ml.
Glycerol	11.6ml.
Bromophenol blue	10mg.
Distilled water	50ml.

Preparation of samples for SDS-PAGE

50 μ l of samples containing 10-50 μ g of protein were mixed with 50 μ l of 2x sample buffer. The mixture was then held in a boiling water bath for 2 min., cooled and 10 μ l of the sample was loaded on the gel. Samples were stored frozen at -20°C.

Procedure

10% solution of polyacrylamide was prepared in the resolving buffer. SDS was added to a final concentration of 1%. A 8.5cm. x 7cm. X 0.75 mm. thick vertical resolving gel was prepared by pouring the slurry between an aluminium oxide and a glass plate separated by a 0.75mm thick spacer. A separate 5% polyacrylamide solution was prepared in stacking gel buffer containing 1% SDS and the solution was poured to form a layer of stacking gel 1.5cm x 8.5cm. x 0.75 mm

above the resolving gel. Ten sample channels were prepared on top of the gel with a comb.

10 μ l of samples containing 1-5 μ g of; test protein and known protein weight markers in sample buffer containing 2% SDS and 5% 2-mercaptoethanol were loaded on the gel. Both upper and lower reservoirs of the electrophoresis tank were filled with reservoir buffer containing 1% SDS. Electrophoresis was carried out at 25mA constant current for about 1h (till Bromophenol blue is just off the bottom of the gel). This gel was then stained by silver staining technique of Blum *et al* (527).

Silver Staining

Reagents

a) Fixative solution

Methanol	50ml.
Acetic acid, glacial	12ml.
Formaldehyde, 37%	50 μ l.
Distilled water	38ml.

b) Pre-treatment

Sodium thiosulphate	0.02gm.
Distilled water	100ml.

c) Staining solution

Silver nitrate	0.2gm.
Formaldehyde, 37%	75 μ l.
Distilled water	100ml.

d) Developer

Sodium carbonate	7.5gm.
Formaldehyde, 37%	75 μ l
Sodium thiosulphate	0.5mg.
Distilled water	100ml.

e) Destainer solution

Methanol	5ml.
Acetic acid, glacial	12ml.
Distilled water	38ml.

Procedure

- | | |
|--|---|
| <u>Fixation</u> | : The gels were fixed in the fixative solution overnight. |
| <u>Washing</u> | : Gels were washed in 50% ethanol in distilled water for 20min. This was repeated thrice. |
| <u>Pre-treatment</u> | : Gels were pre-treated with sodium thiosulphate for exactly 1min. |
| <u>Washing</u> | : Gels were rinsed in distilled water for 20sec. This was repeated thrice. |
| <u>Silver impregnation</u> | : Gels were treated with silver nitrate solution for 20min. on a shaker. |
| <u>Developing</u> | : Gels were developed in developer solution for 5 min. (until the bands were developed). |
| <u>Washing</u> | : Gels were then washed thoroughly with distilled water for 2 min. This was repeated twice. |
| <u>Termination of development</u> | : Staining was stopped by soaking the gel in the destainer solution. |

II.c) Interleukin 2 assay

The IL-2 content was estimated using the IL-2 dependent, mouse cytotoxic T lymphocyte line (CTLL) as mentioned previously (46). Serial dilutions of ConA sup were made in the medium and distributed 100 μ l/well. To this, 10⁴ CTLL/well were added, volume made to 200 μ l and cultured for 20h, pulsed with thymidine and incorporation of the label assayed 16h later. The dilution of the ConA sup at which, 50% of the maximum incorporation occurred was designated to contain 1 IU/ml of IL-2. From this IL-2 content /ml was computed employing Probit analysis.

II.d) Preparation of IgG fraction from serum

The IgG fraction was separated from whole serum by ammonium sulphate [(NH₄)₂SO₄] precipitation method. One volume of saturated (NH₄)₂SO₄ solution was added slowly to two volumes of serum, keeping the mixture being gently stirred at 4°C. After 30 min. the solution was centrifuged at 10,000g for 30min. The supernatant discarded and the precipitate was resuspended in the buffer of choice. This solution was dialysed against the same buffer extensively to remove SO₄⁻² ions. The absence of SO₄⁻² was tested by BaCl₂ precipitation test. The protein content of the IgG fraction was estimated both by spectrophotometry (extinction coefficient for 1.0 mg/ml solution at 280nm taken as 1.4) and by Lowry's method.

II.e) Standardization of Conjugate dilution

The dilution of conjugate to be employed in a given ELISA was standardised as follows :

1. Solution of the required antigen (for example, mouse IgG for anti-mouse IgG HRP conjugate) was prepared in the coating buffer at concentrations 1 μ g/ml, 5g/ml and 10 μ g/ml.
2. 100 μ g/ml BSA solution in coating buffer was also prepared.
3. 100 μ l of each concentration of antigen added to the wells of ELISA plate (Maxisorp, Nunc) in adequate numbers to titrate various dilutions of the conjugate. 100 μ l of the BSA solution was added to another set of wells also in sufficient numbers to test the various dilutions of the conjugate.
4. Plate was covered and incubated in a moist storage box overnight at 4°C.
5. Next day, the well contents were discarded and the unbound excess coating solution was removed by banging the inverted plate several times onto a pad of paper towelling.
6. All the wells were filled with 2% Gelatin, the blocking solution and incubated at 37°C for 1 ½ h.
7. The plates were washed 5-6 times with saline-tween 20.
8. Three suitable dilutions (such as 1:5000, 1:10,000, 1:20,000) of the conjugate to be tested, were made in dilution buffer.
9. 100 μ l of each conjugate dilution were added to the wells. PBS-Tween 20 was added to a set of wells as conjugate blank and incubated at 37°C for 1h.
10. The plate was then washed 5-6 times and 100 μ l of the substrate solution (TMB + H₂O₂) was added. The plate was kept in dark at room temperature for 10-12 min.

11. When optimal colour developed, the reaction was stopped with 50 μ l of 2M H_2SO_4 and absorbance was read at 450nm in ELISA plate reader.
12. A_{450} values were plotted against the dilution of conjugate.

II.f) Heamagglutination assay for autoantibodies against autologous red cells.

Blood was collected from C3H, normal (B6 x C3H)F1 and those with GVHD for more than 10 weeks from retro orbital plexus and sera was separated. C3H sera was washed with saline and 1ml cell pellet of RBCs were diluted in 50 ml.saline to get 2% RBCs suspension.

In a heamagglutination titre plate following reagents were added.

- 100 μ l of test sera with serial dilution till 1:128.
- 100 μ l of 2% RBCs of C3H in all the wells.
- 50 μ l of (1:10 diluted)antiIgM (raised from pooled normal mouse IgM)

Plate kept at 37°C for 24h and checked for heamagglutination pattern.

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SYNOPSIS



INTRODUCTION

Effector response and memory are two major outcomes of exposure to antigen. Tolerance, anergy and clonal loss due to apoptosis are the important other outcomes of antigenic exposure (1). Events that lead to these latter outcomes decide the size of effector and memory response. In addition to these, when one considers the size of effector and memory response of a given functional type (such as T helper response), it is also dependent upon simultaneous activation of functionally irrelevant clones capable of recognising and competing for the same antigenic epitope/s and lymphokines required for growth and differentiation (2).

In this study these two important factors that decide the outcome of immunization in terms of size of effector and memory response of CD4⁺ T helper (Th) cells have been investigated. The factors investigated are :-

- 1) Events of activation that induce clonal loss and thus reduce the size of memory and effector pool.
- 2) Factors leading to activation of functionally irrelevant CD8⁺T cells capable of abrogating or reducing the size of CD4⁺ Th cell memory and effector response by competing for antigen and/or lymphokines required for growth and differentiation.

Factors affecting activation.

In 1970, Bretscher and Cohn proposed a two signal model of lymphocyte activation (3) to explain varied outcomes following immunisation such as clonal expansion, and effector generation and unresponsiveness. The model in its modern form is as follows -

In order to induce an antigen specific immune response the lymphocyte clone must receive a signal delivered by antigen through its clonally selected receptor, the T cell receptor or the surface immunoglobulin. The first signal delivered by antigen is not adequate for full activation. A second signal, qualitatively different and temporally following the antigenic signal is also essential for activation (4). Signal two is derived from diverse sources. In case of CD4⁺ T cell, it is the lymphokine IL-2, generated by the cell itself. In case of CD8⁺ T cells, also the second signal is IL-2, but the source is not autologous (5). In case of B cells, the second signal is delivered by several lymphokines such as IL-4,5,6 (6).

Over the years considerable advances in our understanding of the two signals have taken place. It is now accepted that the first signal itself may not be delivered through the single receptor for antigen. It is known that the antigen receptor signal is coupled to signals delivered from several other non clonally selected receptors, such as CD3, CD28, CD4/CD8, adhesion molecules in case of T cells and α , β chains, CD40, CD19, CD20, CD21, CD22, MHC class II etc for B cells (7,8).

Bretscher and Cohn also proposed that inappropriate delivery of two signals results into unfavourable consequences such as tolerance and anergy. Even though, considerable information is available, significant lacunae still exist in our understanding of lymphocyte activation and differentiation, and factors that decide the size of memory and effector pool. Knowledge of these would be of immense help in understanding several clinical situations associated with abnormalities of activation related clonal anergy/loss.

We have employed an experimental model, that would enable us to study this two signal paradigm. The model used is "Chronic Graft versus Host disease".

Chronic Graft versus Host disease (GVHD)

Graft versus host disease is induced by injecting parental lymphocytes into F1 animals of two inbred strains (9). When the injected parental cells are low in CD8+T cells, the precursors of cytotoxic T cells, a characteristic chronic disease syndrome is shown by the recipients which is known as chronic GVHD (10). This is the consequence of allogeneic response of parental CD4+ T cells to MHC class II antigens of the other parent expressed on the F1 cells (9). Chronic alloantigenic stimulation of parental CD4+T cells classically results into secretion of large amount of lymphokines. The animals harbouring chronic GVHD show in early stages, inability to mount self MHC restricted CD4+T cell response to foreign antigens. Yet the cells respond to polyclonal mitogen normally. This is a typical example of aberration of response to immunisation in terms of size of effector and memory pool.

Hence, the evolution of memory and effector response of CD4+T cells after immunisation with *Mycobacterium vaccae* was studied in this model. The effector response was assayed by delayed type hypersensitivity (DTH); assays of lymphokine secreted by primed spleen cells on stimulation by antigen and memory response by lymphoproliferation test.

The second issue affecting the outcome of immunisation namely factors leading to activation of CD8+T cell clones capable of competing with CD4+ Th cells has also been studied. For this we

have employed earlier reported model of CD8+ T suppressors (11). In this model, immunisation by intradermal (i.d.) route with a killed vaccine of *M.vaccae* generates both DTH and lymphoproliferation responses (effector and memory responses respectively). In contrast intraperitoneal (i.p.) immunisation fails to generate these. The latter effect is due to simultaneous activation of CD8+T suppressors (Ts) capable of competing with CD4+T cells for IL-2. In this study, the factors that are made available for activation of CD8+Ts on i.p. immunisation are investigated.

The issues discussed here have important implications. The information has relevance in vaccine design. The information also has relevance in infectious diseases associated with chronic carrier state, where there is activation related non-responsiveness. The information also has relevance to autoimmune diseases, where there is failure of tolerance induction to the alleged self antigen.

Based on these; a study with the following Aims & Objectives was undertaken.

- 1) Establish an experimental chronic graft versus host disease in mice.
- 2) Study generation of effectors and memory response of CD4+T helper cells in them.
- 3) Study factors affecting the size of suppressor CD8+T cell response.

Materials and Methods

Animals : 8-10 week old C57BL/6 (B6) and C3H/J mice obtained from National Institute of Health, USA and maintained at Cancer Research Institute, Bombay were used in the study. (C57BL/6X C3H/J)F1 -F1(B6F1) and (C3H/J X C57BL/6)F1- (C3HF1) were raised in the laboratory. Prior to immunisation all mice were tested for DTH response to the sonicate of *M.vaccae*; only those that showed no response were used in the experiments.

Chronic Graft versus Host Disease (GVHD) Induction : Normal C3H/J mice were sacrificed, spleens harvested and single cell suspension made 5×10^7 spleen cells were injected intravenously (i.v.) in B6F1 animals to induce GVH.

Assessment of GVH reaction by GVH Index : was done by method the of Simonsen (12). Normal animals and those with chronic GVHD were sacrificed, spleens removed, weighed and the spleen weight was expressed as a fraction of body weight.

The spleen weight : Body weight ratio of the experimental animals was compared with those of normal mice and expressed as GVH index.

$$\text{GVH Index} = \frac{\text{spleen wt / Body wt of each test F1}}{\text{Mean Spleen wt / Mean Body wt of normal F1}}$$

The above ratio, when greater than 1.34 was considered as an index of GVHD.

Microorganism : *Mycobacterium vaccae* was kindly supplied by Dr. J. L. Stanford of Middlesex Hospital, London. The

strain was maintained on L.J. slants and cultivated on the medium of Doubs and Youmans (13).

Immunisation : A one week old culture of *M. vaccae* grown on the medium of Doubs and Youmans, was killed by irradiation (2.4 Mrad) in a Co60 source. A vaccine containing 10⁸ cells in 0.05ml PBS for i.d. immunisation was prepared as described earlier (14). The i.d. immunisation was given in flanks. For i.p. immunization the same number of organisms were suspended in 0.5ml PBS.

Preparation of Sonicate : Mycobacterial cells were subjected to 2.4 Mrad gamma radiation in a Co60 radiation source, washed and were disrupted by sonication (Branson sonifier, B30), sonicate centrifuged at 50,000 g for 1 hr. to remove the cell debris. The supernatant was collected, filtered through 0.22 μ m membrane filter (Millipore, U.S.A.) and its protein content estimated by Lowry's method (15). After confirming sterility the solution was aliquoted and stored at -40°C and used as test antigen.

Delayed type hypersensitivity testing (DTH) : Delayed type hypersensitivity testing was done by the method of Gray and Jenings (16). Briefly a 0.03 ml volume of the sonicate containing 2mg/ml of protein was injected into the hind foot pad and measurement of foot pad thickness was taken before, 24 hr and 48 hr after injection. The results were expressed as corrected foot pad enlargement (CPE), which is the difference between the foot pad enlargements, caused by the test antigen, in the immunised and control mice, tested simultaneously. Results were analysed by students T test, by comparing the CPE values with zero.

Lymphoproliferation assay : This was carried out employing standard microculture technique (17). Briefly each well of the microculture plate received 2×10^5 cells in 0.2ml of culture medium RPMI 1640, containing 2.0mM L-glutamine; 5% FCs and 1% AA and requisite amount of antigen or mitogen were added for stimulation. These are given in detail in each experiment. Cultures were pulsed 120h later for antigen and 72h later for mitogen with $1 \mu\text{Ci}$ of ^3H labelled thymidine for a period of 18h, harvested and incorporation of the label in the cellular DNA assayed. All tests were run in triplicates.

Separation of T cells : was done by the method described by Julius et al (18).

Irradiation of spleen cells for use as antigen presenting cells (APCs) : Spleen cells of mice were subjected to 800 rad gamma radiation in a Co60 source. The irradiated cells were washed in RPMI 1640 and used as APCs.

Lymphokine assay : Lymphokines IL-2, IL-4 and IL-10 in culture supernates were assayed by ELISA employing commercial kits (Genzyme and Pharmingen).

Results and Discussion

Size of the clonal burst, induced by antigen, consequently the size of the memory and effector pool derived from a clone as indicated earlier, is subject to diverse factors, two of which are being studied in this investigation

- A) Effect of excessive/altered activation signals on the size of the response and
- B) Efficiency of activation of competing clones, that are capable of controlling the size of the desired clonal expansion by antigen.

A) Study of effect of excessive/altered activation signals on the size of the response to an antigen

This problem was approached using a chronic GVHD model, in which one can expect to have up graded antigen presentation function of different cell types such as dendritic cells, B cells and macrophages. The alteration in the latter can be induced by cytokines secreted by parental T cells recognising the other parental alloantigen on F1 cells.

1. Establishment of Chronic GVHD in mice

Chronic GVHD was induced in B6F1 mice by transfer of 5×10^7 normal spl proliferation.

GVH index in each of the experimental animals was greater than 1.34, proving successful induction of GVH. Everytime GVHD was induced, the GVH index was calculated to ensure that the mice were suffering from chronic GVHD.

2. Immune responsiveness of mice harbouring Chronic GVHD for 3 months

8-10 weeks old B6F1 mice were injected 5×10^7 C3H spleen cells i.v. to induce chronic GVHD. After 3 months, mice were immunised with killed 10^8 M.vaccae cells by the i.d. route, known to be immunogenic (19). Normal B6F1 mice were also immunised as controls. 7 days later mice were tested for T cell responses namely the DTH and the in-vitro lymphoproliferative response by stimulating the spleen cells with 20ug/ml sonicate of M.vaccae. in-vitro polyclonal T cell responses of the animals to Con A (10,5,2.5 ug/ml) was also simultaneously tested.

Normal B6F1 mice mounted a good DTH and lymphoproliferative responses to M.vaccae. Con A response was normal. Animals with chronic GVHD of 3 months duration showed a poor DTH response. The lymphoproliferative response to M.vaccae sonicate was also very poor. The proliferative response to polyclonal mitogen con A, was depressed though not as severely as the response to antigen.

3. Immune responsiveness of mice harbouring GVHD for 3 weeks

8-10 week old B6F1 mice were given 5×10^7 C3H cells i.v. to induce chronic GVHD as before. After 3 weeks the mice were immunised with 10^8 killed M.vaccae by the i.d. route and their DTH and lymphoproliferative responses against M.vaccae sonicate tested one week later.

Mice harbouring GVHD for 3 weeks showed a good T cell response on immunisation with M.vaccae, as indicated by DTH and lymphoproliferation.

These two experiments indicated that chronic GVHD of 3 months duration results into loss of self MHC restricted T cell responsiveness as reported earlier (10). However, such a T cell response to antigen is preserved in animals having chronic GVHD of 3 weeks duration only.

4. Effect of repeated immunisation on T cell responsiveness to M.vaccae in mice harbouring chronic GVHD for 3 weeks

Chronic GVHD was induced in B6F1 mice as before and after 3 weeks these mice were immunised with 10⁸ killed M.vaccae i.d. The immunisation was repeated twice more at 7 days interval and DTH tested 7 days after the last immunisation. Lymphoproliferative assay was also done with varying numbers of primed splenic T cells using syngeneic 10⁵ irradiated whole spleen cells from normal B6F1 as APC's and M.vaccae sonicate as stimulating antigen.

Once immunised normal B6F1 and those with chronic GVHD mounted a good DTH response. Thrice immunised normal B6F1 also showed a good DTH response. In contrast, thrice immunised B6F1 with chronic GVHD showed a poor DTH response.

Lymphoproliferative response also showed a similar pattern. Normal B6F1 mice showed a good in-vitro proliferative response following 1 dose or 3 dose immunisation. However, in mice with chronic GVHD repeated immunization resulted into loss of responsiveness.

Hence it was concluded that repeated immunization of mice with chronic GVHD results into loss of responsiveness to the antigen.

Based on these results, one may hypothesize that the loss of responsiveness observed at three months could also be the consequence of activation of the lymphoid cells occurring naturally, by the antigens in the environment, by self antigens (cross-reactive) or by idiotypes.

In GVH reaction it is known that parental T cells are activated by MHC class I and II antigens of the other parental strain presented on F1 cells and hence one can think of GVH disease to be the consequence of this basic pathology.

So far as chronic GVHD is concerned parental CD4+T cells may achieve this through (a) Lymphokines or (b) Inducing changes in the APC's or both. Hence the role of these two in the induction of non-responsiveness was investigated.

5. Lymphokines secreted by the effector T cells of mice having chronic GVHD for 3 weeks and repeatedly immunised with M.vaccae

It can be argued, that the observed non responsiveness on repeated immunization of B6F1 mice with chronic GVHD, is not a true non-responsiveness of the animals, but a shift of their response to TH2 types.

Hence B6F1 mice having chronic GVHD of 3 weeks duration and normal B6F1 were given 3 i.d. immunizations with killed M.vaccae. One week after the last immunisation the mice were sacrificed their spleen cells cultured, with or without M.vaccae sonicate, for 48h after which the supernates were harvested for IL-2,

IL-4 and IL-10 assay by ELISA employing commercial kits from Pharminagen, U.S.A.

Normal mice generated only IL-2, even on repeated immunization. In contrast, mice with chronic GVHD did not generate IL-2. Significant amounts of IL-4 and IL-10 were present in the culture supernatants. But these lymphokines were present even in cultures not stimulated by antigen indicating that these were being produced due to GVH reaction and not due to specific antigenic response.

Lymphokine secretion pattern of splenic T cells on stimulation by Con A were also studied in chronic GVHD of 3 months duration. Normal spleen cells generated only IL-2. In contrast spleen cells of mice with chronic GVHD generated IL-4 and IL-10 also. This Lymphokine profile is that of memory T cells (20).

Since in chronic GVHD, T cells recognise the other parental alloantigen and mount an allogenic response, the above lymphokines could have been secreted by the parental T cells. Alternatively, the above lymphokine profile is shown by memory T cells. In combination with loss of ability to mount self MHC restricted response to foreign antigens, the above lymphokine profile is suggestive of loss of naive CD4⁺T cell clones. Since the spleen cells of repeatedly immunized B6F1 mice with chronic GVHD failed to generate any lymphokine on in-vitro stimulation, it was concluded that the observed non-responsiveness was not a TH2 shift of the host response.

6. Effect of Cyclosporin A on the loss of responsiveness observed in chronic GVHD mice on repeated immunizations

Cyclosporin A is known to inhibit activation and inhibit the secretion of lymphokines by T cells (21). Hence it was decided to employ pulse therapy of Cyclosporin A and study its effect in chronic GVHD mice on repeated immunizations.

B6F1 mice having chronic GVHD of 3 weeks duration were given 0.5mg Cyclosporin A per mouse intraperitoneally for 5 days 0,1,2,3 and 4. On day 1 the mice were immunized with 108 killed M.vaccae by i.d. route. After every 10 days the treatment of Cyclosporin A and i.d. immunization were repeated twice again. One week after the third immunization mice were tested for DTH and lymphoproliferative assay performed on spleen cells.

Chronic GVHD mice repeatedly immunized alongwith the pulse treatment of Cyclosporin A did mount a good DTH and lymphoproliferative responses. The chronic GVHD mice which were not given Cyclosporin A treatment, but repeatedly immunized showed emergence of antigen specific non responsiveness as before.

Based on these results it was concluded that the non-responsiveness emerging in chronic GVHD mice due to repeated immunization was related to activation of T cell clones recognizing the antigen, namely M. vaccae. Normal mice on repeated immunization did not reveal any such non-responsiveness. One of the possible mechanisms by which this can happen is activation induced clonal loss or apoptosis.

One of the known models of activation induced clonal loss is that of superantigen induced polyclonal stimulation (22). In this model, in-vivo stimulation with a superantigen such as Staphylococcal enterotoxin B (SEB) results into loss of T cells expressing TCR with VB8, due to apoptosis (23). Hence it was decided to investigate the extent of superantigen mediated activation induced clonal loss in chronic GVHD and compare it with that occurring in normal mice.

7. In-vitro proliferative response to SEB of spleen cells of chronic GVHD mice treated with SEB.

Normal B6F1 mice and those with chronic GVHD were given an ip injection of 0.25 ml/mouse containing 25 ug of SEB. Two weeks later their spleen cells were harvested and their proliferative response to SEB (10ug/ml) was studied, in-vitro. For comparison, cells of untreated mice were also similarly stimulated with SEB.

As expected SEB treated mice with or without chronic GVHD showed reduced in-vitro response to SEB. However decline in responsiveness of mice with chronic GVHD was far greater. Since decline in responsiveness has been shown to be due to loss of SEB reactive T cells, this indicated that the degree of T cell loss following stimulation with SEB was far greater in mice with chronic GVHD.

Activation induced clonal loss due to apoptosis has been generally considered to be a physiological event designated to control the size of the clonal burst following immunization and to prevent autoreactive clones from mounting a pathological immune response to self antigens (24). Excessive activation has been

hypothesised as one of the mechanisms inducing apoptosis. In this context pulse therapy with Cyclosporin A around the time of immunization can reduce the extent of activation of T cell clones recognising the specific antigen and hence rescue them from apoptosis. Indeed this was observed in our model of loss of responsiveness due to repeated immunization of mice with chronic GVHD.

In T cell stimulation, both specific signals delivered through TCR and co-stimulatory signals delivered through CD28 and other adhesion molecules play an important role in activation (25). In chronic GVHD, the cytokines secreted by parental T cells on activation by allogeneic antigens on F1 APC's can induce changes in a number of cells which can behave as APC's namely the B cells, Macrophages and Dendritic cells. As a consequence the later when presenting antigen, can theoretically deliver excessive specific and non specific signals by virtue of upgradation of antigen processing, presentation and expression of co-stimulatory molecules.

8. Antigen presentation by spleen cells of mice with chronic GVHD.

In order to confirm the above possibility efficiency of presentation of antigen by the APC's of mice with chronic GVHD was compared with that of normal mice.

10^5 i.d. primed T cells of normal B6.F1 mice were cultured with different numbers of irradiated spleen cells of normal mice or those with chronic GVHD of 3 weeks duration to serve as APC's & stimulated with different concentrations of M.vaccae sonicate as antigen.

The irradiated spleen of chronic GVHD mice induced a much lower proliferation response.

From this it can be concluded that, the T cells recognising the antigen on APC's of chronic GVHD mice suffer from much higher level of apoptosis as compared to the T cells recognising the same amount of antigen on normal APC's.

Hence it can be concluded that the observed, activation induced clonal loss in chronic GVHD could be due to enhanced activation and exhaustive differentiation brought about by changes in the APC's due to chronic GVHD.

Conclusion

1. Chronic GVHD of 3 months duration revealed loss of self MHC restricted response to foreign antigens, mediated by CD4+T cells. Lymphokine profile of Con A stimulated spleen cells of these animals resembled that of memory T cells.
2. These two findings suggests major decline in the levels of naive CD4+T cells in the peripheral lymphoid organs of mice with chronic GVHD of 3 months duration.
3. Mice with chronic GVHD of 3 weeks duration mounted CD4+T cell response to *M.vaccae*. However, repeated immunization induced non responsiveness.

The non responsiveness was due to clonal loss induced by immunization.

Similar clonal loss was observed on stimulation with superantigen also.

4. Antigen presenting cells of mice with chronic GVHD were abnormal. Both splenic and thymic APC's induced a very poor 5 day lymphoproliferative response.
5. These findings indicate that the major decline in naive CD4+T cells in the periphery is likely to be the consequence of
 - a) Excessive negative selection in thymus
 - b) Excessive apoptosis of peripheral CD4+T cells activated by self antigens, environmental antigens, idiotypes etc.

These phenomena arise predominantly as a consequence of alterations in the APC's of the animal, due to lymphokines secreted by parental T cells recognising the F1 alloantigen.

B) Efficiency of activation of competing clones, that are capable of controlling the size of the desired clonal expansion by antigen

This issue is being investigated employing an earlier reported model of antigen specific immune suppression mediated by CD8+T suppressor (TS) cells (11). In this model i.p. immunization of mice results into activation of both CD4+T helpers (Th) and CD8+Ts capable of recognizing the 65-kDa antigen of *M.vaccae*. The later bring about suppression of response of CD4+ Th by competing for IL-2 and thus reduce the size of effector and memory pool of CD4+ Th recognizing the 65-kDa antigen of *M.vaccae* (26).

In this investigation the same model is employed with the objective to study the role of endogeneous or exogenous superantigen in activation and expansion of the CD8+ Ts cell clone recognizing an unrelated bacterial antigen, such as 65-kDa antigen of *M.vaccae*.

For this, we have employed C3H, B6 and the two sets of F1 mice, C3HF1 and B6F1. These strains of mice were chosen to get varying combinations of IE [the MHC class II molecule preferably employed by most superantigens for interaction (27)] and infectious mouse mammary tumor virus (MMTV) superantigen expression in the different strains. C3H are IE positive chronic carriers of MMTV; B6 mice are IE negative and do not have MMTV infection. C3HF1 mice, are like C3H mothers, namely they express both IE and have MMTV infection and B6F1 express IE but do not have MMTV infection.

1. Pattern of CD4+TH cell responses in the four strains of mice immunized with M vaccae.

C3H, C3HF1, B6 and B6F1 mice were immunised with 10^8 killed M.vaccae by i.d. or i.p. route and were tested for CD4+ Th responses, namely DTH. 7 days later, mice were also sacrificed and the proliferative responses of their spleen cells assayed.

Mice of all the four strain showed a good DTH response on i.d. immunization; i.p. immunization generated a poor DTH response in C3H and C3HF1 mice.

B6 and B6F1 mice showed a definite DTH response on i.p.immunization also, though the foot pad swelling was less than that of i.d. immunized mice.

Similar pattern was observed in lymphoproliferative responses. Thus it appeared, as if i.p. immunization generated suppression in C3H and C3HF1 mice but failed to generate suppression in B6 and B6F1 mice.

2. Adoptive transfer of suppression by ip primed cells.

The C3H and C3HF1 mice which do not mount a response on i.p. immunization were sacrificed and the ip primed spleen cells (whole and L3T4 or anti LYt 2 plus complement treated) were transferred to syngeneic recipients. The latter were immunized by i.d. route and their DTH tested one week later. The i.p. primed CD8+T cells of both the strains were found to be able to suppress the response of the recipients.

3. Unusal behaviour of i.p. primed B6F1 spleen cells.

B6F1 mice were immunized by i.p. route and 7 days later, 5×10^7 cells were transferred into B6F1, C3H and C3HF1 mice. Recipients were then immunized by i.d. route and tested for DTH response 7 days later.

B6F1 recipients mounted a good DTH response where as the C3H and C3HF1 mice showed poor DTH response. Hence it can be said that i.p. immunization of B6F1 mice do expand CD8⁺ T cells. However the size of the suppressor pool is inadequate to abrogate the animals own response. On transfer to C3H or C3HF1 mice the same cell preparation expands adequately and abrogates the response of the recipients. Hence it was concluded that C3H and C3HF1 mice must be providing some factor that enhances the response of CD8⁺T cells following immunization.

4. B6F1 Mice can be induced to mount suppression on i.p. immunization by preimmunization transfer of irradiated normal C3H or C3HF1 spleen cells.

In order to test the above hypothesis stated in section 3, 5×10^7 irradiated whole spleen cells from C3H or C3HF1 mice were injected i.v. in B6F1 and the recipients were immunized by i.d. or i.p. route subsequently, 7 days later their DTH response was tested.

Such a cell transfusion had no effect on i.d. immunization. In contrast, the recipients failed to mount a response on i.p. immunization.

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Such a cell transfusion had no effect on i.d. immunization. In contrast, the recipients failed to mount a response on i.p. immunization.

C3H and C3HF1 mice are MMTV carriers. Hence it was hypothesized that they may be providing some additional factor that facilitates CD8+T cell expansion following ip immunization. The only possible factor that can be provided by C3H and C3HF1 mice would be the MMTV superantigen. Since superantigen interactions are IE dependent, poor CD8+Ts cell response on ip immunization of B6 mice is significant.

Conclusions

1. Immunization of most out-bred and in-bred strain of mice generated suppression mediated by CD8+Ts cells.
2. Ts cell activation was dependent upon expression of IE and superantigen of MMTV.
3. Based on this it was concluded that MMTV superantigen enhances the CD8+Ts cell - antigen interaction by linking the lateral surfaces of TCR-VB and MHC-class II.

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