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Staphylococcal Enterotoxin B Induced Clonal Anergy in αβ T Cell Receptor Transgenic Mice

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ABSTRACT. Superantigens such as staphylococcal enterotoxin B (SEB) have been widely used to study T cell tolerance. While both clonal deletion and apparent anergy have been described in response to superantigens, it has not been clear whether true anergy occurs, or whether the most reactive T cells are selectively deleted, leaving less reactive clones. To address this question, the response of an essentially monoclonal population of primary T cells to a superantigen was followed by priming ovalbumin (OVA) specific αβ T cell receptor transgenic mice with SEB. Cells from these mice appeared to be anergic in that they were hyporesponsive to OVA peptide as well as to SEB. The anergic cells could respond to PMA and ionomycin suggesting that a proximal signal transduction step was affected. Cells from transgenic mice primed with OVA peptide, the specific antigen, were not anergic. Thus, in this system, the ability to tolerize mature T cells appears to be a property unique to superantigens. To address the question of whether superantigen induced tolerance was due to T cell activation in the absence of costimulation, we examined the effect of the CD28/B7 pathway on the response of T cells to SEB and OVA. The T cell response to both OVA and SEB was enhanced by antibodies to the CD28 coreceptor suggesting that CD28/B7 mediated costimulation plays a role in the signal transduction pathways coupled to the two types of antigens.

Chapter 1.1 (Introduction)

The variable region of T cell receptor is made up from the products of five gene segments from the variable (V), diversity (D), and joining (J) gene families; Vα and Jα make up the α chain, whereas Vβ, Dβ, and Jβ, contribute to the β chain (reviewed in 1). Each chain is generated by the random rearrangement of these non contiguous gene segments (1). Because this is a random process, there is nothing to prevent the generation of T cells with receptors specific for self antigens. Tolerance can be defined as the process by which potentially self reactive lymphocytes are silenced. There have been three major hypotheses proposed to explain how the immune system aquires tolerance to self antigen. They are as follows:

1. Clonal deletion. This theory was first proposed by Lederberg (2) as a modification of Burnet's clonal selection theory (3). The theory proposed that immature cells react differently from mature lymphocytes in response to the engagement of their receptors: immature cells would be deleted whereas mature cells are activated. Thus lymphocytes with self reactive receptors would eventually come into contact with self antigen as they developed and this would result in their selective deletion.

2. Clonal anergy. In this view, self reactive cells were not physically eliminated. Instead they were functionally inactivated or anergized. Evidence for this hypothesis came from studies demonstrating that B cells in fetal mice could be tolerized without being physically deleted (4).

3. Suppression. This hypothesis suggested that self reactive lymphocytes were held in check by suppressor cells or suppressor factors. This hypothesis was supported by experiments showing that when lymphocytes from animals tolerized to a given antigen were transferred into naive animals,they prevented the hosts' lymphocytes from reacting to the tolerizing antigen (5,6).

It has been very difficult to distinguish between these hypotheses in T cells because it has not been possible to physically identify a primary T cell that was specific for any given antigen. Instead, T cell antigenic specificity was defined functionally by a response to the antigen. Thus, in the absence of a response, one could not determine whether self reactive cells had been deleted or were present but not reactive due to either anergy or suppression. The discovery of superantigens which activate clonal populations of T cells has therefore had major implications in the study of T cell tolerance.

There are two major groups of confirmed superantigens, toxins from different bacteria including staphylococci and streptococci, and proteins encoded by the endogenous and exogenous mouse mammary tumor viruses.

The best way to describe a superantigen is to compare it with the so called conventional antigens. Before a conventional extracellular protein antigen can activate a T cell it must first be processed into peptides by antigen presenting cells (7) which are then presented in a specialized cleft present in the major histocompatibility complex (MHC) class II proteins on the cell surface. The bacterial toxins are also proteins but they apparently do not have to be processed prior to presentation; in fact pretreating toxins with proteases tends to reduce the response to these antigens (8). The toxins also tend to bind differently to MHC class II molecules; they do not appear to bind to the peptide cleft since mutations in this region affects T cell responses to peptides but not to superantigens (9).

The most distinctive feature of a superantigen is the manner in which it is recognized by T cells. The trimolecular interaction between conventional peptide, MHC, and TCR involves to some extent all five gene segments of the variable region of the TCR, especially the hypervariable region made up from the junctional segments (10). Because of the specificity of this interaction, very few T cells will react to any given conventional antigen. Superantigens, on the other hand, interact primarily with the Vβ gene product (11-13) and can therefore activate T cells in a Vβ specific manner. Because this interaction requires only Vβ which may be shared by a large number of T cells bearing

different specificities for conventional antigens, many more T cells will respond to a superantigen than to a conventional antigen. There are approximately 20 different Vβ families in the mouse and it thus follows that any given superantigen will activate roughly 5% of the entire T cell repertoire.

The viral superantigens were first described by Festenstein as undefined antigens present in certain strains of mice that would induce powerful immune responses in other mice even if the two strains had the same MHC haplotype (14). He named these antigens minor lymphocyte stimulatory (Mls) antigens. It has since been shown that Mls and Mls like antigens are encoded for by the open reading frame (ORF) present in the 3' long terminal repeat sequence of the endogenous and exogenous mouse mammary tumor viruses (MMTVs) (15-21).

The sequences of these viral superantigens are very similar except for 20-30 residues at the carboxy terminal which determine the specifity for Vβs (22-25). In vitro translation studies have shown that the ORF proteins are type II integral membrane proteins with the carboxy terminus present at the cell surface (26-28). This correlates with the finding that the carboxy terminus of the viral superantigens determine Vβ specificity, as this region would be expected to interact with T cells. Monoclonal antibodies (mAbs) have been recently developed to the ORF protein of MMTV and have been shown to block T cell activation (29-31). In one study the mAb was used to demonstate that the superantigen was expressed on the surface of activated B cells but not on resting spleen cells, thymocytes, or activated T cells (29). In addition it was shown that the superantigen probably exists on the cell surface as a processed 18.5 kilodalton protein.

Because superantigens stimulate T cells primarily through the Vβ element of the TCR, the development of mAbs to different Vβ segments enabled investigators to physically identify superantigen reactive T cells and this revolutionized the study of tolerance. In fact the first direct evidence for clonal deletion came from work done with superantigens.

Clonal deletion

Kappler et al. demonstrated that >90% of all hybridomas tested that expressed Vβ17a as part of their TCR would respond to an endogenous superantigen (most likely an MMTV) presented by the MHC class II I-E molecule (32). This superantigen has still not been mapped but it appears to be present in all strains of mice (33), is presented exclusively by the I-E molecule, and is expressed in B cells but not in macrophages or fibroblasts (34).

Kappler et. then used a mAb they developed to Vβ17a to compare $I\text{-}E^+$ and $I\text{-}E^-$ strains of mice. They reasoned that since the I-E molecule (and the endogenous superantigen it presented) was a self antigen, $I\text{-}E^+$ mice should be tolerant to this molecule. This turned out to be the case; peripheral Vβ17a T cells were present in $I\text{-}E^-$ mice but not in $I\text{-}E^+$ mice (35). Furthermore, when $I\text{-}E^-$ mice were mated to $I\text{-}E^+$ mice, the F1 mice had no mature Vβ17a T cells. When these F1 mice were mated to $I\text{-}E^-$ mice, it was found that all the F-2 mice that had inherited the I-E molecule had low levels of Vβ17a cells whereas their $I\text{-}E^-$ littermates had normal levels of these cells (35).

On comparing the thymuses of $I\text{-}E^-$ and $I\text{-}E^+$ mice, it was found that both sets of mice had roughly equal numbers of immature cells expressing low levels of Vβ17a. $I\text{-}E^+$ mice however, had almost no mature Vβ17a thymocytes whereas $I\text{-}E^-$ mice did (35). Thus it appeared that immature thymocytes expressing a self reactive receptor were not allowed to complete development and leave the thymus.

This was strong evidence for intrathymic clonal deletion and it opened up a whole new field of using superantigens to study tolerance. Similar techniques were used by Kappler et al. to demonstrate that Vβ8.1 T cells reacted to the endogenous superantigen Mls-1 (36). This antigen has since then been shown to be the product of mtv-7 (18). MacDonald et al. showed that this same superantigen reacted with Vβ6 cells (37), and both groups demonstrated deletion of these Vβ types in $Mls\text{-}1^+$ (Mls-1a) but not $Mls\text{-}1^-$ (Mls-1b) mice. Another Mls antigen, Mls-2 ,was shown to interact specifically with

Vβ3 cells and deletion of these cells was demonstrated in Mls-2$^+$ (Mls-2a) mice (38,39).

Clonal anergy

In addition to clonal deletion, clonal anergy has been shown to be an important mechanism for inducing tolerance. Much of the initial work in this field resulted from work done with bone marrow radiation chimeras. These chimeras are based on the finding that cells derived from the bone marrow, such as lymphocytes and dendritic cells, are relatively susceptible to radiation whereas thymic stromal epithelial cells are relatively radioresistant. Mice can therefore be irradiated to eliminate their bone marrow derived cells and reconstituted with cells from a different genetic background.

Two groups independently made chimeras where the Mls-1 and I-E associated superantigens were present only on the radioresistant thymic epithelial cells (40,41). Vβ6 and Vβ17a cells, derived from the bone marrow of Mls1b and I-E$^-$ donor mice, were not deleted but appeared to be functionally tolerant as they did not proliferate in response to Mls-1a, I-E$^+$ spleen cells in vitro. In addition to this, the cells did not respond to immobilized anti Vβ6 or Vβ17 mAbs implying that these cells were anergic. Interestingly, the addition of exogenous IL-2 could partially reverse anergy, suggesting that IL-2 secretion was a major defect in these cells.

This is very similar to the in vitro clonal anergy described by Ron Schwartz and colleagues. In that model, anergy is induced in T cell clones by engaging the TCR in the absence of a second or costimulatory signal (42). This two signals versus one signal hypothesis was proposed by Bretscher and Cohn as an explanation for B cell tolerance (43). In the most physiological variation of this model, planar membranes containing purified MHC class II molecules pulsed with specific peptide were shown to induce a long lasting state of anergy in T cell clones (44). The costimulatory signal appears to be important for the secretion of IL-2 which acts as an autocrine growth factor that is essential for T cell proliferation.

It was argued that the anergy seen in the bone marrow chimeras was due to the fact that the thymic epithelial cells could provide the first signal (in this case the superantigen and MHC class II) but not the second costimulatory signal. This hypothesis was supported by earlier work by Lorenz and Allen, where it was shown that thymic epithelial cells could present peptides to hybridomas but could not induce proliferation in T cell clones, implying that they were lacking an essential costimulatory signal (45).

T cell receptor transgenic mice

In order to study tolerance in a system with a relatively homogenous population of T cells, several investigators generated β and αβ T cell receptor (TCR) transgenic mice and then looked at their response to endogenous superantigens. Pircher et al made TCR transgenic mice, >90% of whose T cells expressed the Mls-1 reactive Vβ8.1 segment, and then crossed them onto an Mls-1a background (46). They found that lymph nodes (LNs) from these mice contained one third the number of cells found in Mls1b mice. The remaining cells generated diminished cytotoxic T lymphocyte (CTL) responses to allogeneic target cells. The response could be partially restored by the addition of cuture supernatant from concanavalin A (Con A) stimulated T cells (presumably containing IL-2 and other cytokines). The CTL response to vaccinia virus infected target cells was also reduced but surprisingly, the mice could produce T cell dependent neutralizing IgG to vesicular stomatitis virus just as efficiently as Mls-1b transgenic mice. It is therefore likely that the T cells were defective in producing lymphokines needed for a CTL response but could produce factors needed to help B cells.

Blackman et al. generated similar Vβ8.1 TCR transgenic mice and bred them onto Mls-1a and Mls-1b backgrounds (47). They found almost no deletion of CD4 cells and no deletion of CD8 cells in Mls-1b mice. Many (20-50%) of all CD4 cells from Mls-1a mice appeared to be pan anergic in that they did not respond well to Mls-1a spleen cells, staphylococcal enterotoxin B (SEB, a bacterial toxin superantigen that stimulates $V\beta8^+$ T cells), anti TCR mAbs, or concanavalin A. CD8 cells were not anergic in that they

proliferated as well as cells from Mls-1b mice. Interestingly mature CD4 thymocytes were not anergic, implying that cells were anergized in the periphery.

The genes for the α and β chains from a lymphocytic choriomenigitis virus (LCMV) specific CD8 T cell clone were used to make αβ TCR transgenic mice (48). The β chain was Vβ8.1 and therefore the T cells were specific for Mls-1a as well as LCMV. When these mice were bred on an Mls-1a background, 65% of the mature CD8 cells were deleted. The remaining cells were not anergic and could proliferate in response to LCMV infected peritoneal macrophages and specifically lyse LCMV infected target cells. This might correspond with the finding of Blackman et al. that CD8 T cells were not anergized by Mls (47).

In contrast to the moderate degree of tolerance seen in response to Mls, the investigators demonstrated that when the mice were infected with LCMV at birth, there was a marked deletion of immature thymocytes and a 90% reduction in the number of peripheral CD8 T cells (48). Only one third of the remaining CD8 T cells expressed the transgenic β chain and there was no proliferative or CTL response to infected cells. It therefore appeared that the conventional antigen was better able to induce tolerance than the superantigen, but this could be due to either a dose effect or to a greater affinity of the original clone for its specific antigen than for the superantigen.

Berg et al. generated both β and αβ transgenic mice using transgenes from a hybridoma specific for cytochrome c (49). The β chain was Vβ3 and therefore reactive to Mls-2. Both sets of transgenic mice were bred on Mls+ and Mls- backgrounds. In the Mls-2a transgenic mice, less than 10% of the LN cells were $V\beta3^+$ compared to the 70-90% found in Mls- transgenic mice. The investigators did not report the number of peripheral T cells present in the Mls-2a αβ transgenic mice but there was much more intrathymic deletion in these mice than in β transgenic mice due to increased TCR expression. No functional tests were performed so it is not known if the remaining cells in either line were anergic.

MMTV transgenic mice

When it had been shown conclusively that the Mls and Mls related superantigens were encoded for by endogenous MMTV, two groups made mice transgenic for exogenous MMTVs to further study the role of these viruses in T cell development. In one study, transgenic mice expressing either the entire genome of mtv(GR) or just the ORF present in the 3' long terminal repeat were made (21). This MMTV is located on chromosome 2 of GR mice but is capable of producing infectious virus. The superantigen produced by this virus stimulates Vβ14 T cells. The mice used in the study did not have an endogenous equivalent of this MMTV and therefore had normal numbers of Vβ14 cells. Mice expressing either transgene deleted their Vβ14 cells. This was direct evidence that the MMTVs were responsible for Vβ specific deletion in mice. Interestingly mice transgenic for the mutant virus MMTV C3H-K did not delete their Vβ14 cells. Compared with GR, this mutant virus has several point mutations as well as substitution of LTR sequences resulting in a major difference in the last 32 residues of the ORF gene. This finding correlates with the observation that the C terminal residues determine Vβ specificity.

In a similar study, Golovkina et al. made transgenic mice expressing the ORF from MMTV C3H (50). This virus is transmitted through milk (17) and the ORF present in the 3' LTR has been shown to encode for a superantigen specific for Vβ14 and Vβ15 T cells(20). It was found that mice expressing this transgene deleted their Vβ14 cells and that in different transgenic lines the degree of deletion correlated with the level of transgene expression. Interestingly, mice expressing high levels of the ORF transgene did not become infected with the exogenous virus present in their mother's milk. This was evidence that the exogenous virus utilized Vβ14 T cells in its infectious pathway (probably to transmit the virus to the mammary gland) and suggested that by deleting specific Vβ populations, endogenous viruses may help protect mice from infection with exogenous viruses that had similar superantigen specificities.

Cell types involved in Mls mediated deletion

These transgenic models were useful in demonstrating tolerance to endogenous superantigens but they did not address the question of which cells were actually involved in tolerance induction. Before the exact nature of the Mls superantigens was known, several investigators performed adoptive transfer studies where specific subpopulations of cells from Mls+ donors were transferred into Mls- hosts.

Webb and Sprent injected into neonatal Mls-1b mice highly purified CD4 cells, CD8 cells, B cells or T cell depleted spleen cells (51). The mice were then examined two to seven weeks later for signs of tolerance to Mls-1. CD8 T cells were 50-100 times more efficient than CD4 T cell or B cells in inducing clonal deletion of Vβ6 cells and in inducing tolerance to Mls -1a spleen cells. This was an unexpected finding since CD8 T cells do not express MHC class II antigens. It was assumed that either Mls antigen was transferred from CD8 T cells to traditional antigen presenting cells (APCs) or that there might be corecognition of Mls present on CD8 T cells and MHC class II molecules on APCs.

In similar experiments, Inaba et al. injected either B cells or splenic dendritic cells from Mls-1a mice directly into the thymuses of Mls-1b neonatal mice (52). Thymic but not splenic B cells were tolerogenic in that they were able to delete Vβ6 thymocytes and induce functional tolerance as measured by a graft versus host reaction. Interestingly dendritic cells were able to induce functional anergy but not clonal deletion of Vβ6 thymocytes. This finding confirmed prior studies that had shown that B cells and not dendritic cells were the major presenters of Mls in vitro (53, 54).

Mazda et al. used a different system to adress the same question. Fetal thymus organ cultures were used to culture precursor thymocytes from Mls-1b mice in deoxyguanosine treated Mls1-b thymuses (this treatment specifically kills bone marrow derived cells) in the presence of different Mls-1a APCs (55). It was found that neither B cells nor dendritic cells alone could induce clonal deletion of Vβ6 thymocytes. Efficient deletion was only seen when Mls-1a B cells were injected with Mls-1b dendritic cells.

This raised the possibility that the Mls antigen was being transferred from B cells to host dendritic cells which could then mediate clonal deletion. Dendritic cells from Mls-1a mice probably could not induce deletion by themselves simply because they did not have sufficiently high levels of the Mls antigen.

Intrathymic tolerance to a bacterial superantigen

Jenkinson et al. used a similar fetal thymic organ culture system to demonstrate that that SEB could induce the specific deletion of Vβ8 thymocytes (56). Apoptosis or programmed cell death was implicated as the mechanism of cell death as oligonucleosomal DNA fragments were found in SEB treated but not control cultures. The same group later showed that dendritic cells were much more efficient than thymic epithelial cells in inducing SEB mediated clonal deletion (57).

White et al. demonstrated that the bacterial toxins could induce intrathymic tolerance in vivo as well as in vitro (58). Neonatal mice were injected with SEB every other day from birth to day 15. A partial deletion of immature thymocytes as well as an almost complete deletion of mature thymocytes expressing SEB reactive Vβs was seen. It was also shown that this same toxin would activate mature splenic T cells in vitro. It thus appeared that triggering T cells at different stages of development had different consequences; mature T cells were activated whereas immature thymocytes were deleted. This work was also the first direct evidence that a foreign antigen could induce tolerance in neonates by causing the deletion of reactive T cells.

Peripheral tolerance

In all of these models, the tolerizing superantigen was either expressed in the thymus or could somehow be transported to the thymus. While these models were crucial in defining the mechanims involved in intrathymic tolerance, they did not address the question of how T cells became tolerant to antigens that were expressed only outside the thymus. Several investigators designed experiments using superantigens to address this question of peripheral tolerance.

1. Tolerance in athymic mice

Two groups independently compared the T cell repertoires of Mls+ nude mice to euthymic mice of the same strain (59,60). Nude mice have an epithelial defect which prevents cells in the third and fourth pharyngeal pouches from developing into the thymic anlage. In spite of not having a functional thymus, these mice develop low numbers of αβ T cells in the periphery. It was found that nude mice but not euthymic controls had significant percentages of T cells with Vβs specific for Mls and I-E associated superantigens. It therefore appeared that a thymus was essential for proper clonal deletion of self reactive T cells. Despite the presence of T cells with potentially self reactive receptors, nude mice do not suffer from autoimmune diseases. A later study revealed that T cells from nude mice could not respond to the bacterial toxin staphlococcal enterotoxin A (SEA) (61). This superantigen specifically stimulates Vβ3 and Vβ11 cells. Both of these Vβs also react to endogenous superantigens and are deleted in euthymic but not athymic mice (59,60). The inability of these Vβs to react with SEA suggested that they were probably anergic.

Neonatally thymectomized mice were also used as a model to study peripheral tolerance. Like nude mice, these mice had high percentages of T cells with self reactive Vβs (62), but unlike nude mice they eventually develop a wide array of autoimmune disease (63). It was thus felt that the self reactive T cells might be responsible for the observed autoimmunity. However work by Jones et al. demonstrated that the T cells bearing self reactive receptors were anergic in that they could not proliferate in response to immobilized Vβ specific mAbs (64). The two models suggested that while clonal deletion did not occur in the absence of a thymus, peripheral anergy existed as a backup mechanism of tolerance induction.

2. I-E transgenic mice

In a somewhat different approach, Lo et al. made transgenic mice expressing the I-E α and β chains only in non lymphoid organs. The rat insulin or the rat elastase promoter

were used to target the transgenes to pancreatic β (65) or acinar cells (66) respectively. T cells from both sets of mice appeared to be tolerant to I-E$^+$ spleen cells and no T cell mediated autoimmune disease was seen.

There is some question as to whether the transgenic I-E molecules were presenting endogenous superantigens or allogeneic peptides. The endogenous superantigen presented by the I-E molecule stimulates T cells bearing the Vβ17a segment. Whereas no deletion of Vβ17a cells was seen in either line (65,66), T cells from β cell transgenic mice but not from acinar cell transgenic mice were tolerant to immobilized anti Vβ17 mAb (67). It thus appeared that the tolerance seen in the acinar transgenic mice was to allogeneic peptides and not to the endogenous superantigen. A possible explanation for the difference seen in the two transgenic lines is that acinar cells might not express the endogenous superantigen and as such T cells in these mice may have never been exposed to it.

There is also some question regarding the leakiness of both promoters. Although transgenic mRNA was not seen in the thymuses of either line, the thymocytes were surprisingly tolerant to I-E$^+$ spleen cells (65,66). Therefore these mice may not be a good model for peripheral tolerance as one cannot be sure at what stage the T cells first came into contact with the antigen in question.

3.Peripheral tolerance to Mls

In a different model, Rammensee et al. injected Mls-1a spleen cells into adult Mls-1b mice (68). Surprisingly, T cells from the recipient mice became tolerant to the Mls-1a spleen cells; they could neither proliferate nor secrete IL-2 in response to these cells in vitro but could respond normally to third party alloantigens. Anergy appeared to be the mode of tolerance as there was no deletion of Mls-1 reactive Vβ6 cells, and suppression was ruled out by cell mixing experiments. The anergic Vβ6 cells were able to express the IL-2 receptor α chain when stimulated with Mls-1a spleen cells but the addition of exogenous IL-2 did not restore proliferation. Thus it appeared that Mls could tolerize mature T cells as well as thymocytes. It should be noted that in the previously described

models of peripheral tolerance, the T cells devloped in the presence of the tolerizing antigen. In this model however it is significant that T cells that had matured in the complete absence of the Mls antigen were rendered tolerant after their initial contact.

Webb et al. performed a similar experiment but used thymectomized mice in order to keep the peripheral T cell repertoire stable (69). Purified Mls-1a B cells, CD8 T cells, and T cell depleted splenocytes were all able to tolerize Mls-1b mice. However in contrast to Rammensee et al., the investigators observed a marked expansion followed by partial deletion of $V\beta6^+$ CD4 cells; these cells expanded from roughly 10% to 40% by day 4 and then decreased to 2-3% by day 22. Viable cells were required to induce the observed tolerance as irradiated Mls-1a cells failed to induce either clonal deletion or a decrease in the proliferative response to Mls-1a spleen cells. This was the first direct evidence of clonal deletion of mature peripheral T cells and the authors concluded that tolerance could be the endpoint of a strong immune response.

Peripheral tolerance to Staphylococcal enterotoxin B

Two groups independently demonstrated that the the bacterial superantigen SEB could also induce anergy in mature T cells (70,71). In one study, adult mice were immunized intravenously with different doses of SEB. CD4 cells but not CD8 cells were found to be anergic to the superantigen in vitro (70). The degree of anergy was proportional to the dose of SEB administered. CD4 cells were defective in IL-2 production and proliferation, but surprisingly, they were able to specifically lyse SEB pulsed target cells in CTL assays (70). Rellahan et al. demonstrated that cells from SEB primed mice were non responsive to immobolized Vβ8 mAb as well as to SEB (71). It was found that Vβ8 cells were able to express the IL-2 α receptor in response to SEB, but exogenous IL-2 did not restore proliferation (71). This finding was similar to the results of experiments of Ramennsee et al. on Mls induced peripheral anergy, and suggested that there might be a defect in IL-2 responsiveness as well as IL-2 secretion.

It was later found that the in vivo response to SEB was quite similar to the response to Mls; there was an initial clonal expansion followed by partial deletion of Vβ8 T cells (72,73). Two independent studies demonstrated that when cells at the clonal expansion phase were cultured overnight at 37 C, the Vβ8 cells declined to control levels (72,73). One study suggested that programmed cell death was involved in deletion, as genomic DNA fragments (although not in an oligonucleosomal pattern) were seen in Vβ8 but not in Vβ6 cells (72).

The presence of DNA fragmentation in cells from SEB primed mice was confirmed by another group (74), but it was found that inhibitors of protein and RNA synthesis failed to prevent this DNA fragmentation, significant because these agents have been shown to prevent some forms of apoptosis in cell lines. Only zinc, which blocks activation of endonucleases and aurintricarboxylic acid, which directly inhibits endonuclease activity, could prevent this DNA fragmentation (74). DNA fragmentation is also seen in cells from Mls-1b mice primed with Mls-1a cells (75) but in both models the cells first have to be cultured in vitro at 37 C. DNA fragmentation is never seen in cells taken ex vivo immediately from superantigen primed mice implying that either apoptotic cells are cleared very rapidly in vivo, or apoptosis might not really be the mechanism of cell death in vivo.

The reversibility of anergy

1. The role of time/ persistance of antigen

Migita and Ochi recently demonstrated that SEB induced anergy is gradually reversed with time (76). Cells from thymectomized mice primed with SEB four months prior to sacrifice could proliferate in response to SEB just as efficiently as cells from control mice. The investigators used Thy1.1/Thy1.2 chimeras to rule out the possibility that the observed proliferation was due to the selective outgrowth of a population of non anergic cells. The investigators however did not determine if the IL-2 secretion response was restored as well.

This reversal of anergy with time may be due to the gradual loss of SEB in vivo as Ramsdell and Fowlkes have shown that the persistence of superantigen is required for the maintenance of anergy (77). Using the previously described bone marrow radiation chimera (40), it was found that taking T cells (derived from the bone marrow of Mls-1b, I-E^- mice) from the irradiated Mls-1a, I-E^+ hosts and "parking" them into irradiated Mls-1b, I-E^- mice reversed anergy by 20 days post transfer. Specifically the T cells could now respond to immobilized anti Vβ6 and Vβ17 mAbs. The same effect was seen when purified T cells were cultured in vitro in the presence of interleukin-7 as a trophic factor.

2. The role of IL-2

Lack of IL-2 production has been shown to be a major defect in anergic T clones. T cells from mice treated with either SEB (70) or Mls+ cells (68) produce significantly less IL-2 than control cells in response to the respective superantigen. The addition of exogenous IL-2 does not fully restore proliferation (68,71) suggesting that other defects are present.

IL-2 however does appear to at least partially reverse anergy of potentially self reactive T cells in nude mice. In one study it was shown that these cells would undergo clonal expansion in response to the bacterial superantigen SEA only in the presence of IL-2 (61). Based on this work, Gutierrez-Ramos et al. treated nude mice with either a recombinant vaccinia virus expressing the human IL-2 gene or wild type vaccinia virus as a control (78). It was found that T cells with self reactive receptors in the mice treated with the recombinant virus were now able to proliferate in response to immobilized Vβ specific mAbs and the mice developed such signs of autoimmunity as erythroleukopenia, rheumatoid factors and autoantibodies to double stranded DNA. No autoimmune disease or reversal of T cell anergy was seen in mice treated with the wild type virus. While there is no evidence that the activation of the self reactive T cells was

responsible for the observed autoimmune disease, this study clearly demonstrated that IL-2 could reverse some forms of anergy when given in vivo as well as in vitro.

In contrast to the results seen with nude mice, treating SEB primed mice with the same recombinant virus did not reverse anergy (74). In fact it had no effect at all on the kinetics or magnitude of either clonal expansion or deletion. This finding suggests that not all forms of peripheral anergy have the same underlying defect, and it correlates with the inability of exogenous IL-2 to reverse SEB induced anergy in vitro (71).

3. Infection with parasites

Rocken et al. infected either control or SEB primed mice with Nipostrongylus brasiliensis, a nematode that induces T cell proliferation and interleukin-4 (IL-4) secretion in vivo (79). It was found that infection resulted in an increase in the absolute number of Vβ8 T cells in the mesenteric LNs of both control and SEB treated mice implying that the anergic cells were capable of proliferating in response to the nematode. Furthermore, CD4 cells from both sets of infected mice produced high levels of IL-4 in response to in vitro challenge with either SEB or immobilzed Vβ8 mAb. Cells from infected SEB primed mice however could still not secrete IL-2 in response to either mitogen. While it appears that the nematode was able to partially reverse SEB induced anergy, it is also possible that a subset of non anergic Vβ8 cells proliferated in response to the nematode and differentiated into Th-2 type cells which can secrete IL-4 but not IL-2.

Factors affecting the degree of deletion or anergy

1. Cyclosporin

Vanier and Prud'homme injected different doses of the immunosuppresive drug cyclosporin A (CsA) into thymectomized BALB/c (Mls-1b) mice that had been primed with either SEB or with Mls-1a spleen cells (80). They found that high doses of the drug (50mg/kg/day i.p.) enhanced Vβ specific deletion in both models. Paradoxically, it was found that although mice treated with SEB and CsA had slightly fewer Vβ8 T cells than

mice treated with SEB alone, cells from these mice were not anergic to SEB. In other words the coinjection of CsA appeared to 1) increase the degree of Vβ8 T cell deletion and 2) abrogate anergy to SEB. This prevention of anergy but not the increased Vβ specific deletion was still seen when lower doses of CsA (> 12.5mg/kg/day) were given.

It is difficult to understand why CsA would on one hand enhance clonal deletion and yet on the other abrogate anergy. These results would suggest that deletion and anergy may be independent of each other and have completely different mechanisms of actions. CsA blocks IL-2 secretion and has effects on other cytokines as well (81). It is interesting that a drug that blocks IL-2 secretion would prevent rather than enhance anergy, a state associated with decreased IL-2 production. It should be noted that in a prior study by another group, comparable doses of CsA had no effect at all on either deletion or anergy in SEB treated mice (74).

2. Cortisone

Lusssow et al. demonstrated that hydrocortisone could enhance the SEB mediated deletion of Vβ8 T cells in thymectomized mice (82). Furthermore Vβ8.1 TCR transgenic mice bred on an Mls-1a background deleted almost all of their remaining T cells when they were treated with cortisone. This effect was not seen in Vβ8.1 TCR Mls-1b transgenic mice or in control mice. This work suggested that mature T cells that had interacted with a superantigen but had escaped deletion were susceptible to cortisone whereas control T cells were not.

3. Bcl-2 oncogene

The bcl-2 protooncogene encodes a protein that appears to be associated with the inner membrane of mitochondria (83). It has been found that the overexpression of this gene prevents the death of certain cell lines in response to the withdrawal of growth factors (84). Transgenic mice have been made that express bcl-2 under the regulation of an immunoglobulin enhancer (85). Some strains of these transgenic mice express bcl-2 in T cells as well as B cells. Thymocytes from these mice survive longer than normal

thymocytes in response to agents such as sodium azide, dexamethasone and γ irradiation (86).

When these mice were primed with SEB, Vβ8 peripheral T cells expanded to levels that were 1.5-3 fold higher than found in SEB primed non transgenic mice (86). The activated Vβ8 T cells did not decline as rapidly or to the same extent as did cell from non transgenic mice. Interestingly, the bcl-2 transgene had no effect on anergy induction. This might suggest that anergy and clonal deletion have two different mechanisms and that the bcl-2 oncogene plays a role in one but not the other. Alternatively, it might mean that anergy and deletion are two steps on the same pathway, and that bcl-2 is involved in a step somewhere after anergy induction but before clonal deletion.

Role of other TCR elements in superantigen reactivity

Vacchio et al. designed experiments to determine whether the Vα segment contributed to the reactivity of Vβ6 cells to Mls-1 (87). Four Vα specific mAbs were developed and each was used in two color flow cytometric (FACs) analysis with the Vβ6 mAb. T cells from Mls-1b mice were then incubated with Mls-1a stimulator cells. As expected there was a clonal expansion of Vβ6 T cells in response to Mls-1 but somewhat suprrisingly, there was a skewing of the Vα subtypes present on the Vβ6 cells. In C3H and BALB/c mice, there was a consistent decrease in the percentage of Vβ6 T cells expressing either Vα2 or Vα 8. In B10.A mice, there was a relative increase in the percentage of Vβ6 cells that were $V\alpha11^+$ and relative decreases in cells that were either $V\alpha3^+$ or $V\alpha8^+$. No such skewing of Vα subtypes was seen in response to immobilized Vβ6 mAb. This work suggested that Mls recognition was influenced by Vα expression as well as by Vβ expression.

Smith et al. further demonstrated that α chains contribute to superantigen reactivity. The investigators generated 176 hybridomas from Vβ8.1 mls-1a TCR transgenic mice and 120 from Vβ8.1 Mls-1b TCR transgenic mice (88). They then determined the Mls-1 reactivity of each hybridoma and correlated this with the Vα gene expressed. Of the

hybridomas from Mls-1b mice, 43% were Mls-1 reactive with a good correlation between Mls reactivity and the Vα gene expressed. In particular, Vα11.1 cells were generally Mls-1 reactive whereas Vα2, Vα8 and Vα11.3 cells were generally not Mls-1 reactive. Furthermore there was a skewing of Vα usage in hybridomas generated from Mls-1a transgenic mice. The non Mls reactive Vα2 and Vα8 hybridomas were overrepresented whereas Mls reactive Vα 11 cells were completely absent in these mice.

Almost all of the hybridomas from both sets of mice were SEB reactive, but the dose required to induce one half the maximal stimulation varied by more than a thousand fold. This is relevant since decreases observed in SEB induced anergy usually ranges from 50 -90% and could therfore be easily be explained by the selective deletion of Vβ8 cells with highly reactive Vα subunits.

This Vα selective deletion has now been demonstrated in vivo. Vβ8.1 TCR transgenic mice were treated with SEB and hydrocortisone to induce deletion. A comparison between these mice and untreated controls revealed skewing of the Vα repertoire; there was a marked increase in the percentage of Vα2 cells and a decrease in the percentage of Vα8 T cells (89).

Taken together, these studies can be interpreted to suggest that priming mice with SEB may result in the selective deletion of highly reactive T cell clones and that this selective deletion rather than true clonal anergy might account for some or most of the diminished response to SEB.

Because this point is essential, one of the main goals of this project was to distinguish between selective deletion versus true clonal anergy. To do this we collaborated with Dr. Dennis Loh at Washington University to obtain αβ TCR transgenic mice expressing transgenic TCRs specific for an ovalbumin (OVA) peptide in the context of I-A^d (90). Both transgenes were obtained from the DO11.10 hybridoma (91) which responds to OVA 323-339 (92). The β chain is Vβ8 and therefore the cells respond to SEB as well as to OVA, the specific antigen. A clonotypic mAb, KJ1-26, has been developed to this

hybridoma. T cells in the DO11.10 transgenic mice that express both α and β TCR transgenes can be detected with this clonotypic mAb (90) and should all be equally responsive to SEB.

As will be shown in the Results section, when these mice were primed with SEB, diminished responses to OVA, SEB, and immobilized clonotypic mAb were seen even though the number of KJ1-26 cells present was comparable to that found in control mice. This is consistent with true clonal anergy. The anergic cells were able to respond to PMA and ionomycin, suggesting that a proximal signal transduction step was affected. This step however, is probably not calcium mobilization as anergic cells could mobilize calcium in response to Con A.

To determine whether conventional antigens could also induce tolerance, the transgenic mice were primed with OVA peptide. Cells from these mice responded normally to SEB and were hyperresponsive to OVA, suggesting that, at least in this system, superantigens but not conventional antigens can tolerize mature T cells.

Chapter 1.2 (Materials and Methods)

Transgenic mice. An 8 week old agouti male, heterozygous for the DO11.10 α and β transgenes, was received from Dr. Dennis Loh (Washington University, St. Louis). DO11.10 mice from Dr. Loh's laboratory were maintained on an H-2^d background and were routinely mated to BALB/c mice.

The male was placed into a specific pathogen free (SPF) room at Rockefeller University's Laboratory Animal Research Center (LARC) and acclimatized for several days. The original male was then mated to four new 8-9 week old BALB/cBy females every week. The females were also kept under SPF conditions and acclimatized for at least a week after arrival at LARC. As soon as they were visibly pregnant, the females were placed into separate cages. Pups from these mice were weaned at 3-4 weeks of age. At the time of weaning, ear markings were made on the left and/or right ear(s) of the mice with an ear punch (National Band & Tag Co., Newport, KY). Thus each individual mouse could be identified by sex, cage card number, coat color (approximately 50% of the pups were white the other 50% agouti), and ear markings.

Since the male was heterozygous for both transgenes, only 50% of its offspring would be TCR transgenic (the two transgenes were injected as a linear construct and therefore always cosegregate). The pups therefore had to be screened for the presence of the transgenes. For screening, genomic DNA was obtained from the tail tips of mice and the polymerase chain reaction (PCR) performed on the DNA using probes specific for the α transgene (since the two transgenes always cosegregate, mice transgenic for the α chain would also be transgenic for the β chain).

Tail tips were taken during weaning, right after the ear markings were made. Approximately 2cm of the tail tip was cut off with scissors, cut into 2 pieces and then placed into an autoclaved 1.5 ml tube containing 0.5 ml of proteinase K buffer. Scissors and forceps were wiped with ethanol pads between mice to prevent cross contamination.

Proteinase K buffer:

0.1 M NaCl	(1 ml of 5 M stock/50ml)
0.01 M EDTA pH 8.0	(1ml of 0.5M/50ml)
0.05M Tris pH 7.5	(2.5ml of 1M/50ml)
1% SDS	(2ml of 25%/50ml), premixed then add
0.3 mg/ml Proteinase K	(15mg/50ml ie. 0.75 ml of stock)

Proteinase K (Boehringer Mannheim, Indianapolis, IN) was resuspended at a concentration of 20 mg/ml in sterile ddH2O. Aliquots were stored at -20C.

Tail tips were incubated in this buffer for 5-6 hrs at 50-55 C in a heating block. 0.5 ml of a 1:1 mixture of Tris equilibrated phenol (United States Biochemical, Cleveland, OH) and chloroform was then added to each tube. A new pipette tip was used for each tube to prevent cross contamination. The tubes were vortexed vigorously and then centrifuged at 11,000 x g for 10 minutes in a microcentrifuge. 0.45 ml of the supernatant was removed and placed into a tube containing 0.5 ml of chloroform. The tubes were vortexed again and centrifuged for 5 minutes at the same speed. 0.4 ml of supernatant was removed and placed into tubes containing 0.8 ml of ice cold absolute ethanol. The DNA was precipitated at -70C for at least 6 hrs but usually overnight. The tubes were then centrifuged for 30 minutes at 11,000 x g at 4 C. The pellets were washed once with ice cold 70% ethanol, centifuged for 5 minutes, and then air dried under a hood. The dried pellets were resuspended in 50 ul of TE and kept at -4 C (DNA samples). This DNA was diluted at 1:10 in sterile ddH20 and used as the template for the PCR.

The sequences of oligonucleotides used for the PCR screens were obtained from the Loh lab:

Vα13 5' CAG GAG GGA TCC AGT GCC AGC 3'

Jα 5' TGG CTC TAC AGT GAG TTT GGT 3'

These oligonucleotides were synthesized by Oligos etc. Inc. (New Haven, CT), resuspended in sterile H2O at a concentration of 1 mM, and kept at -20 C. DNTPs, mineral oil, magnesium stock solution, 10X PCR buffer, and Taq polymerase were all purchased from Perkin Elmer (Norwalk, CT). PCR was carried out in 50 ul reactions in 0.5 ml tubes.

5.0ul 10x PCR buffer

4.0ul of 25mM Mg (2mM)

1.0ul of 10mM dATP (200uM)

1.0ul of 10mM dCTP (200uM)

1.0ul of 10mM dGTP (200uM)

1.0ul of 10mM dTTP (200uM)

0.25ul of 5U/ul Taq polymerase (1.25 U)

0.125ul of 1mM Vα oligo (2.5uM)

0.125ul of 1mM Jα oligo (2.5uM)

36.5ul of ddH2O. premix, add 2 drops of mineral oil then add:

1.0ul DNA (1:10 dilution of DNA sample)

The reaction was carried out in a Coy thermocycler using the following conditions:

5 min at 94 C (to denature template DNA)

(1 min at 94 C, 1 min at 55 C, 1 min at 72) = 1 cycle. Repeated 30 times.

10 min at 72 (to allow completion of DNA products)

The reactions were then kept at 4 C until a gel coud be run. Products were analysed by agarose gel electrophoresis and staining with ethidium bromide. To analyze the products. 25ul of each reaction was mixed with 5 ul of 6X loading buffer and run on a gel.

2% agarose (Seakem, FMC Bioproducts, Rockland, ME) gels were made in 1X TAE buffer. 1ul/100 ml of a 10mg/ml ethidium bromide solution (Sigma, St Louis, MO) was added

to the gel and to the 1X TAE running buffer. A 100 base pair DNA ladder (Gibco BRL, Gaithersburg, MD) was used as the molecular weight standard. The PCR product was 300 base pairs long and was visualised with U.V. light. When results were equivocal, PCR was repeated with DNA stock solutions diluted at 1:5 as well as to 1:10. All standard stock solutions were made as described in Maniatis.

Nontransgenic mice: Thymectomized BALB/c mice were purchased from Taconic Farms.

Priming mice:

For footpad injections the antigen was first emulsified in complete Freunds adjuvant (CFA, Sigma, St. Louis, MO). 1 ml Hamilton Gastight series teflon fluorocarbon resin luer lock glass syringes (Reno,NV) were used for this process. An equal volume of CFA and the antigen (up to 0.5 ml of each) were taken up into two syringes. The syringes were connected with a three way stopcock (Sherwood Medical, St. Louis MO). The CFA and antigen were then subjected to 100 - 200 cycles through both syringes where 1 cycle represents the complete passage of the suspension from syringe 1 to syringe 2 and then back to syringe 1. Injections were done with these same syringes fitted with 25 gauge needles. The mice were first anesthetized with Nembutal (pentobarbital sodium injection, Abbott laboratories, North Chicago, IL). A 50 mg/ml stock solution was diluted 1:10 with Hanks Balanced Salt Solution (HBSS, Gibco) and 0.3 ml (medium sized mice) or 0.4 ml (larger mice) of this dilution was injected into the peritoneum of mice with a 25 gauge needle. A total of 200 ul of the suspension was injected over all four footpads of each mouse. SEB was purchased from Toxin Technology (Sarasota, FL), resuspended at 1mg/ml in RPMI1640 medium, aliquoted, and stored at -70 C. If 50 ug of the toxin was to be injected into each mouse, the SEB solution was diluted 1:1 with PBS or HBSS . The concentration would now be 0.5mg/ml or 50 ug in 100 ul. This solution was then mixed 1:1 with CFA resulting in a final concentration of 0.25mg/ml or 50 ug in 200 ul.

For intravenous injections, mice were placed in cages without bedding and these cages were then placed onto an electric heating pad (purchased from a local drugstore) for 10-15 minutes. The mice were then suspended through the cage cover racks by their tails and 0.1 ml of antigen was injected into their dilated lateral tail veins with a 25 gauge needle.

Mice were sacrificed with CO_2 usually 10 days after priming. The scissors and forceps used in the dissections and the fur of the mice were first disinfected with 70% isopropanol. Spleens and/or lymph nodes (LN) were taken and placed into a tube containing sterile HBSS. For footpad injections, the axillary, brachial, popliteal and inguinal LNs were harvested and pooled. To extract cells from spleens and LNs, the plunger of a 1 ml syringe was used to force the organs through a 40 or 60 mesh screen held in a "Cellector" tissue sieve (Electrochemical Apparatus, St. Petersburg, FL). Each screen was then washed with 12 ml of HBSS and then the cell suspension [collected in a sterile standard10 x15mm petri dish, (Fisher Scientific)] was centrifuged at 1600 RPM at 4 C for eight minutes. Red blood cells were removed by resuspending the cells into a 1:1 mixture of RPMI 1640 and a 1.66% solution of ammoniun chloride in sterile ddH20. The cells were incubated in 12 ml of this solution (first prewarmed to 37 C) for 3-4 minutes and were then centrifuged again. The cells were then resuspended in HBSS and centrifuged (i.e. washed) twice. Cells were counted in a 10% solution of trypan blue (Gibco) in HBSS with a hemocytometer.

Proliferation assay.

Peptide. OVA 323-339 (ISQAVHAAHAEINEAGR; M.W. 1774) was synthesized and purified by the American Peptide Company (Sunnyvale, CA). The peptide was > 97% pure as determined by HPLC. The lyophilized peptide was stored at -20 C. For each assay a small amount (~0.5 mg) would be weighed out on an analytical balance and a 1mM stock solution of was made in RPMI 1640. Ovalbumin (grade V) was purchased from Sigma and kept at 4 C. For each assay a 10 mg/ml stock solution was made. SEB was

purchased from Toxin Technology (Sarasota FL), resuspended at 1mg/ml, aliquoted and stored at -70 C. Con A (Boehringer Mannheim) was resuspended at 5 mg/ml and was kept at 4 C.

PMA was purchased fom Sigma, resuspended at 1mg /ml in DMSO (Sigma), aliquoted and stored at -20 C. Ionomycin (Calbiochem, San Diego, CA) was resuspended at 10 mg /ml in DMSO, aliquoted, and stored at -20 C.

For the assay cells were resuspended at 10^6 cells/ml in "R+D" culture medium [a 1:1 mixture of RPMI 1640 +DMEM high glucose (Gibco) supplemented with 10% FCS (Hyclone, Logan, UT), 1mM glutamine, 50uM 2-ME, penicillin, streptomycin and amphotericin B (purchased as antibiotic-antimycotic solution, Gibco)] and 200 ul (2 X 10^5 cells) per well dispensed into 96 well flat bottom Corning plates (Fisher). The cells were stimulated with various concentrations of SEB, or OVA 323-339, or with either 100 ug/ml ovalbumin, or 2.5 ug/ml Con A. For receptor cross linking studies, KJ1-26 (2.5 or 5ug/ml in HBSS) or anti CD3 mAb (2 ug/ml; clone145-2C11, PharMingen, San Diego CA) was immobilized onto 96 well plates (100 ul/well) by incubating overnight at 4· C. The plates were washed three times with HBSS before use.

LN $CD4^+$ cells were obtained by passing lymph node cells over CD4 purification columns as per the manufacturer's instructions (Biotex, Edmonton, Canada). The cells were 90-95% $CD4^+$ as determined by FACS analysis. BALB/c spleen cells were cultured at 2 x 10^6 cells/ml in RPMI 1640 medium (supplemented as described above) with 15 ug/ml of LPS (S. Minnesota R595, List Biologicals, Campbell CA) for 3 days. The cells were then inactivated with irradiation (3000R) from a ^{137}Cs source, washed twice and used as antigen presenting cells. 5 X 10^4 CD4 cells + 5 X 10^4 irradiated LPS blasts per well were cultured in the presence of different stimuli as described above.

Splenic $CD4^+$ cells were obtained by treating the cells with anti CD8 mAb. Culture supernatant from the 3.155 hybridoma (ATCC TIB 150) used at a final concentration of 1:5 was incubated with cells suspended in RPMI 1640 at a concentration of 10^7

cells/ml on ice for one hour. The cells were pelleted by centrifugation at 1600 RPM for8 minutes and then resuspended at 4 X 10^7/ml in a 1:10 solution of 4 week old baby rabbit complement (Pel-Freez, Rogers, AR) .The cells were incubated at 37 C for 45 minutes and were then washed two times with RPMI 1640 medium. Cells at this stage were used as "CD8 T cell depleted spleen cells". If purified CD4 cells were needed, the cells were then subjected to panning. Standard10 X 15mm petri dishes, (Fisher) were coated with 5 ml of goat anti mouse anti IgG and IgM antibody (Jackson ImmunoResearch, West Grove PA) in 50 mM Tris pH 7.5 at a final concentration of 20 ug/ml for I hr at room temperature. The plates were then washed four times with HBSS. 5 ml of RPMI with 5% FCS was added and the plates were stored at 4 C until they were ready to be used. A maximum of 10^8 cells in 4.5 ml of RPMI 1640 with 5% FCS was added to each plate and the plates were then incubated at 4 C for an hour. The plates were then gently agitated from side to side and non adherent cells harvested. The plates were then gently washed two times with 5 ml of HBSS. The cells and the two washes were pooled and centrifuged as described.

The cells were then passaged over nylon wool columns. Nylon wool (NW) (Polysciences, Warrington PA) was first untangled with dog brushes or combs and then squeezed into either 5 ml (0.5 gm NW), 10 ml (1gm NW) or 20 ml (2 gm NW) syringes (Becton Dickinson) and autoclaved. 3 X 10^8 bulk spleen cells/gm NW were used. After the colums were washed with RPMI, the plungers were removed and three way stopcocks (Sherwood Medical) were attached to the syringes. RPMI containing 10% FCS, was run through the column and the column was then incubated at 37 C for one hour. The cells were then added in an amount of medium that was just sufficient to enter the column. Another 1 ml of mediun was added and after an hour incubation at 37 C , the cells were eluted at a rate of one drop every 2 seconds. After this step the cells were at least 85% $CD4^+$ as determined by FACS analysis.

These cells were used with A20 cells, an H-2^d B cell lymphoma line from ATCC, (Rockville, MD) or TA3 cells an H-$2^{d,k}$ B cell lymphoma line, or its B7 transfected derivative TA3-mB7 (93). A20 cells were inactivated with 17,000R, TA3 cells were inactivated with 20,000R. 5 X10^4 CD4 cells were incubated with 10^4 lymphoma cells in the presence of different stimuli as described in the Results section. Purified anti-CD28 mAb was purchased from PharMingen.

After either 48 or 68 hrs, 1 uCi of ^{3}H thymidine (Amersham, Arlington Heights, IL) in 15 ul of media was added to each well and the cells were harvested 14 -18 hrs later onto glass fiber filter mats (Pharmacia LKB, Gaithersburg MD) with an automatic harvester (Skatron Inc, Sterling, VA). The filters were dried for an hour in a 37 C oven, placed into a special sample bag (Pharmacia LKB) with scintillation cocktail (Beckman, Fullerton CA) and were then counted with a Betaplate counter (Pharmacia LKB). Triplicates were run for each sample and the mean determined. In some experiments, after 48 hrs, the cells were removed from wells and centrifuged at 1600 RPM at 4 C for 5 mins to harvest the culture supernatant which would then be used in IL-2 assays. The cells were then resuspended in complete medium, redispensed into their original wells, and then pulsed with ^{3}H thymidine.

IL-2 secretion assay.

Cell lines: HT- 2 cells were a gift from Dr. Y. Choi (The Rockefeller University, New York, NY), CTLL-2 cells and concentrated anti interleukin 4 supernatant (clone 11B11, ammonium sulfate precipitate) were from Dr. T. Rao and Len Appleman (NYU School of Medicine). Recombinant mouse IL-2 (Collaborative Research, Belford, MA) and recombinant human IL-2 (Promega, Madison WI), were resuspended at 10,000 units /ml (10 U/ ul), aliquoted, and stored at -70 C.

LN cells at 10^6 cells/ml or 5 X10^5 CD4 cells + 5 X10^4 irradiated (6000R) A20 cells /ml were cultured in R+D medium in the presence of a given mitogen. The cells were cultured in either 24 well flat bottom plates at 1 ml per well, or in 96 well flat

bottom plates at 200 ul per well (proliferation assays). After 48 hrs, the culture supernatants were harvested, reconstituted with medium to the original volume, and kept at - 70 C until use.

CTLL-2 cells were grown in RPMI1640 supplemented with 10% heat inactivated FCS (from Len Appleman), nonessential amino acids, sodium pyruvate, 1mM glutamine, penicillin, and streptomycin (all from Gibco) and 20 U/ml of recombinant human IL-2 . Gentamicin, amphotericin B, and 2-ME were not added as they were toxic to the cells. The cells were passaged twice a week by resuspending 10^6 cells in 20 ml of medium. Cells were used for the assay 3-4 days after they were passaged. They were washed once and then IL-2 "starved" by incubation in medium without IL-2 for 4 hrs before the assay. Cells were resuspended at 6.67 X 10^4 /ml in culture medium, and neutralizing anti IL-4 mAb (1.5 ul/ml) was added. 75 ul (5 X10^3 cells) per well was then dispensed into 96 well flat bottom plates. 4 - 8 serial twofold dilutions of the culture supernatants to be tested were made and 25 ul of the dilutions was added to the cells. 8 -10 serial twofold dilutions of recombinant mouse IL-2 (starting from 1 unit in 25 ul) were also made and added to cells. The cells were pulsed with 1 uci of ^{3} H thymidine in 10 ul of medium after 24 hrs in culture, and were harvested 16 - 20 hrs later as described for the proliferation assay. Triplicates were run for each dilution of each sample and the mean determined.

HT-2 cells were the gift of Dr. Y. Choi (The Rockefeller University), and were maintained and passaged by his laboratory. The cells were used in assays 2 days after they had been passaged. The cells were resuspended at 2 X 10^5 /ml with 5 ug/ml of anti IL4 in 50% modified complete tumor medium (CTM)/ 50% Mishell- Dutton complete cocktail. This mixture consists of a 3:2:1 ratio of modified CTM : nutrient cocktail (NC) : FCS. Modified CTM is DMEM high glucose with glutamine and sodium pyruvate (Gibco) supplemented with 10% FCS (Hyclone), 2 mM glutamine, 50 uM 2-ME, penicillin, streptomicin, gentamicin, amphotericin B, and essential and non essential amino acids

(added just before the assay, all reagents from Gibco). 50 ml of nutrient cocktail was made from 35 ml of MEM medium (Gibco), 5ml of essential amino acids solution (50X), 2.5 ml of non essential amin acids solution (100X) 2.6 ml of glutamine (29.2 mg/ml) [all from Gibco] and 0.5 g of dextrose (Sigma). The pH of the solution was then adjusted to 7.0 with 10 N NaOH and 0.57 g of sodium bicarbonate (Sigma), and 5 ml of ddH20 added. The cocktail was then sterilized by filtration, divided into 2 ml aliquots (which were used only once) and stored at -20 C.

20 ul (4 $X10^3$ cells) was dispensed into each well of 96 well flat bottom plates and 80 ul of culture supernatant or recombinant mouse IL-2 dilutions (made as described above) was added to the cells. 1 uci of ^3H thymidine in 10 ul of medium was added to each well after 18 hrs of culture and the cells were harvested 12 hrs later. For each assay, a standard curve was generated using X (concentration), and Y (thymidine incorporation; CPM) values from the recombinant IL-2 dilutions. These values were then plotted on a Cricket Graph (Computer Associates, Islandia, NY). Those points that fell into a roughly straight line were taken and the equation,

$Y = mX + b$ or

$X = \frac{Y - b}{m}$ where m and b were determined by the program.

was used to determine the concentration of IL-2 present in the culture supernatants

Calcium flux assay: 50 ug aliquots of lyophilized Indo 1-AM were purchased from Molecular Probes (Eugene OR) and stored at - 20 C. Just before each assay one aliquot was reconstituted in 50 ul of DMSO (Sigma). Indo-1 loading buffer (Gibco) contained 5.36 mM KCl, 0.44 mM KH_2PO_4, 136.87 mM NaCl, 4.16 mM $NaHCO_3$, 0.3357 mM $Na_2HPO_4 \cdot 7H_2O$ 11.101 mM Glucose, 5.0 mM HEPES 2.0mM $CaCl_2 \cdot 2H_20$, 0.1% BSA, pH = 7.3. LN cells were suspended at 5 X 10^6 cells / ml in the loading buffer and loaded with 4 ug /ml of Indo 1-AM for 1 hr at 37 C. Cells were washed twice and resuspended at 10^6 cells /ml in loading buffer. The cells were incubated at 37 C for 2 mins before each

experiment. Fluorescence was measured in a spectrofluorimeter (SPF -500C, SLM Aminco Instruments Inc, Urbana, IL) with an excitation of 350 nM and emission recorded at 510 nM. 2 X 10^6 cells were analyzed in spectrofluorimeter cuvettes (SLM Aminco Instruments). After a steady baseline was obtained, the specified mitogen was added. At the end of each experiment, the loaded Indo-1 was released by solubilizing the cells with Triton X 100 (Sigma); 5 ul /ml of a 10% stock solution was added to obtain the maximum fluoresence levels (F max). The calcium chelator EGTA was then added to a final concentration of 5 mM to obtain the minimum fluorescence (F min). Intracellular calcium concentration was determined by the equation:

$(Ca^{2+})i = Kd\ (F - F\ min)/\ (Fmax - F)$

where Kd is the dissociation constant of Indo-1 for Ca^{2+} (250 nM) and F is the observed fluorescence.

FACS analysis: Anti-CD4 PE (clone RM4-5), anti CD8 PE (clone 53-6.7)anti CD44 PE (clone IM7) anti Vβ8.1/8.2 FITC (clone MR5-2) anti αβ TCR FITC and purified anti B^7 were all purchased from Pharmingen. Biotinylated or purified KJ1-26 (obtained from the Loh lab) was used with streptavidin FITC (Pharmingen) or FITC conjugated F(ab)2 goat anti mouse IgG (Fc specific Jackson ImmunoResearch, West Grove, PA) respectively. Anti I-A^d (clone MKD6) and I-E^d (clone 14-44) were culture supernatants from hybridomas purchased from ATCC and were used with FITC conjugated F(ab)2 goat anti mouse IgG. Purified anti CD32 ("Fc block" clone 24G2, Pharmingen) was used to decrease non specific staining. It was not used during staining with unconjugated mAbs as it reacted with the secondary antibodies.

As a general rule, 1 ug of mAb in 100 ul of FACS buffer [1% bovine albumin, (Cohn fraction V, pH 7.4, Sigma) in PBS] or 100 ul of culture supernatant was used to stain 10^6 cells. If 10^5 cells were to be stained, they would be centrifuged at 1600 RPM for 4 minutes at 4 C, and then resuspended in 10 ul of this mAb containing buffer. Cells were usually stained in 5 ml Falcon 2058 tubes (Fisher) for 25 minutes at 4 C and then

washed twice with FACS buffer (1 ml for the first wash, 0.5 ml for the second wash). For secondary staining, FITC conjugated goat anti mouse IgG (Fc specific) or streptavidin FITC was diluted at 1 : 100 in FACS buffer and 30 ul of this solution was added to 10^5 cells. The goat anti mouse IgG cross reacted slightly with many of the rat mAbs, therefore if a 2 color analysis was to be performed with unconjugated KJ1-26 and CD4 PE and/or CD8 PE, the cells would be first treated with KJ1-26, washed twice, stained with FITC conjugated goat anti mouse IgG, washed twice, and then finally stained with the PE conjugated rat monoclonal. The cells were resuspended in 300 ul of FACS buffer and immediately analyzed on a FACScan (Becton Dickinson). In some instances, the cells were fixed with 3.7% formaldehyde solution (37% stock solution (Fisher) diluted 1: 10 in FACs buffer was added to cells at the very last step). Fixed cells were wrapped in foil and kept at 4 C for up to 3 days after staining.

10^4 events per sample were usually aquired. A dot blot of forward scatter versus side scatter was obtained and non viable cells were gated out by size. Dot blots of FL1 (FITC) vs FL2 (PE) were then obtained. Cells stained with only secondary reagents or with isotype matched non specific mAbs were used as controls to determine background staining. Duplicates were run for most samples, the mean determined, and background fluorescence staining subtracted.

Chapter 1.3 (Results)

Induction of tolerance in mature T cells by SEB

In order to confirm published results on the ability of SEB to tolerize mature Vβ8 T cells, BALB/c mice were immunized intravenously with 50 ug of SEB per mouse. As shown in Table I, a clonal expansion of Vβ8 T cells took place by day 3. This is consistent with published reports. By day 10, the Vβ8 T cells were deleted to a level that was slightly lower than that found in control mice (Table I). Vβ 6 cells are not SEB responsive and were largely unaffected throughout the course of the experiment (Table I). The remaining Vβ8 T cells appeared to be functionally tolerant in that they responded poorly to SEB in vitro (Fig. 1a). T cells from these mice could, however respond well to the lectin Con A (Fig. 1b) implying that the observed tolerance was specific for SEB.

In other experiments, SEB was emulsified in CFA and inoculated subcutaneously into thymectomized BALB/c mice. Table II shows that there was a significant decrease in the number of Vβ8 but not control Vβ14 CD4 T cells in the draining lymph nodes of these mice. The remaining cells responded poorly to SEB (Fig. 2a) and to immobilized anti Vβ8.1 + 8.2 mAb (Fig. 2c) but responded as well as control cells in response to immobilized anti CD3 mAb (Fig. 2b). This further confirms that the effect of SEB was specific for a subpopulation of T cells ($V\beta8^+$).

αβ TCR transgenic mice

The next set of experiments were designed to follow the response of a monoclonal population of primary T cells to the superantigen SEB, in mice transgenic for the α and β TCR chains from the OVA specific hybridoma DO11.10. A DO11.10 transgenic mouse and a non transgenic littermate were phenotyped as shown in figure 3. Virtually all $CD4^+$ T cells in both the transgenic mouse and the non transgenic littermate were $CD3^+$. The transgenic Vβ8.2 chain was expressed by virtually all T cells in the transgenic mouse. In contrast only 13% of CD4 cells in non transgenic mice expressed endogenous Vβ8.2 as part of their T cell receptor. The clonotypic mAb KJ1-26 detects the combination of the DO11.10 TCR α and β chains. It follows that only those T cells in

Table I. Percentage of Vβ6 and Vβ8 $CD4^+$ T cells in control and day 3 SEB primed mice.

	Vβ6	Vβ8
Control mice	9.5%	23.1%
SEB primed mice	7.2%	47.5%

Percentage of Vβ6 and Vβ8 $CD4^+$ T cells in control and day 10 SEB primed mice.

	Vβ6	Vβ8
Control mice	12.2%	26.1%
SEB primed mice	12.2%	22.0%

Table II. SEB + CFA immunizations

Percentage of Vβ14 and Vβ8 $CD4^+$ T cells in control and SEB primed mice (day 10).

	Vβ14	Vβ8.1+8.2	Vβ8
Control mice	10.0%	29.0%	19.6%
SEB primed mice	11.1%	12.2%	8.1%

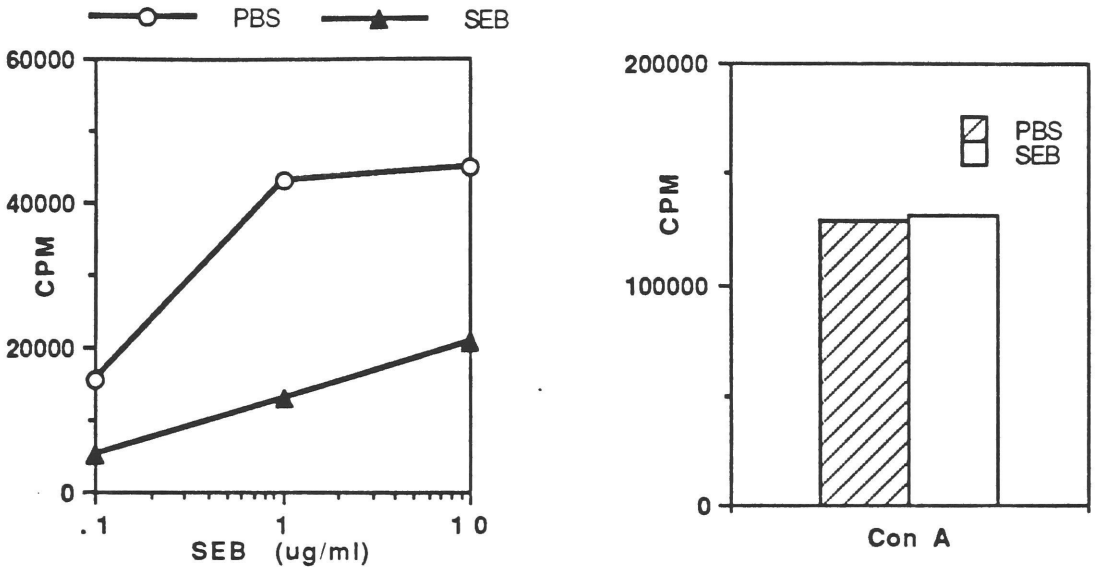


Figure 1. Proliferation of spleen cells from either PBS (open circles) or SEB (closed triangles) primed mice in response to either SEB (a) or 2.5 ug/ml of Con A (b). Cells were pulsed with ^{3}H thymidine on day 3 and harvested on day 4. The mean of triplicates is shown for all experiments.

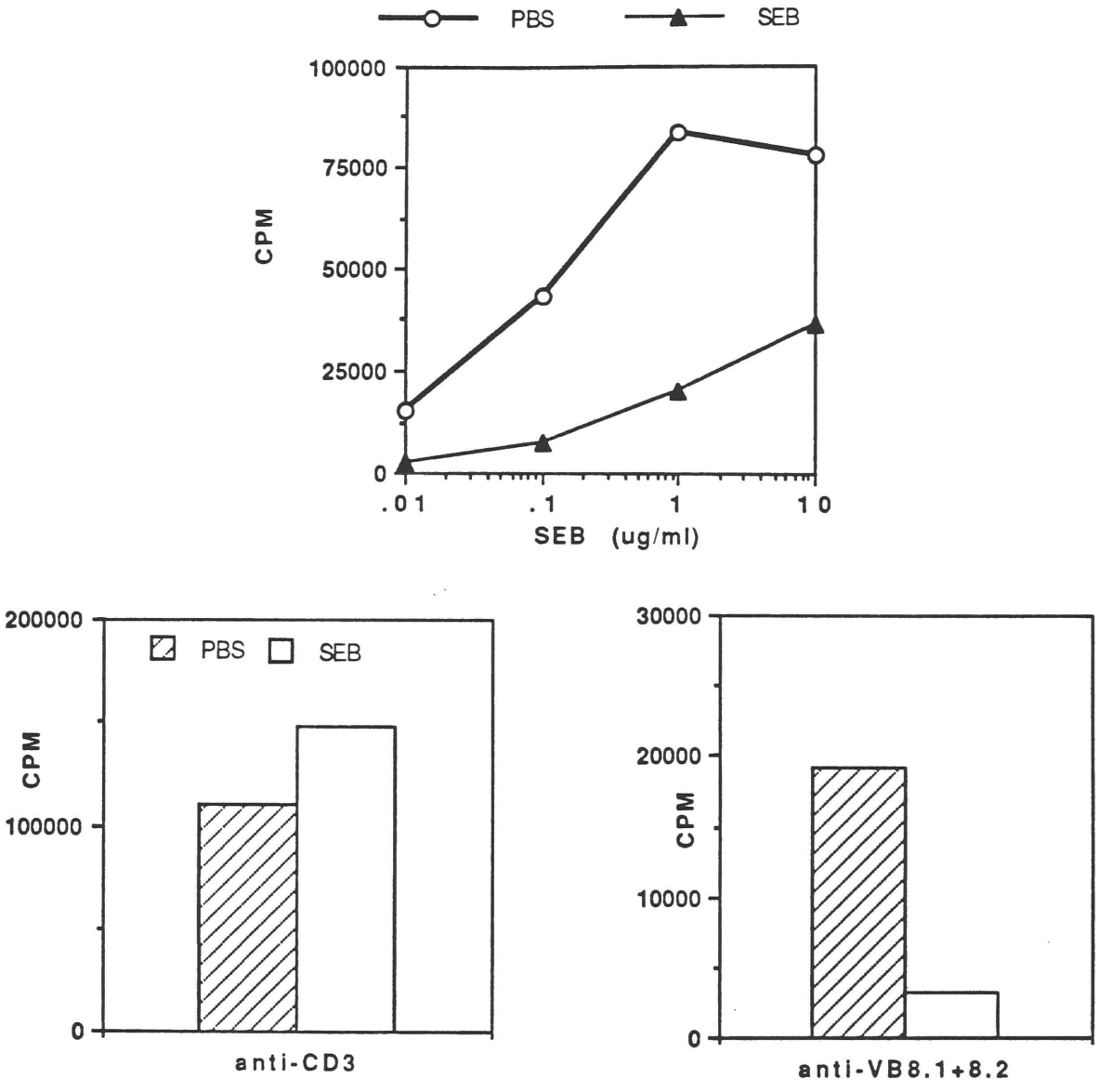


Figure 2. Proliferation of purified CD4 cells from either PBS (open circles) or SEB (closed triangles) primed mice in response to either SEB (top) or 2 ug/ml of anti CD3 (bottom left) or 5 ug/ml of anti Vβ8.1+8.2 (bottom right). Cells were pulsed with ^{3}H thymidine on day 3 and harvested on day 4.

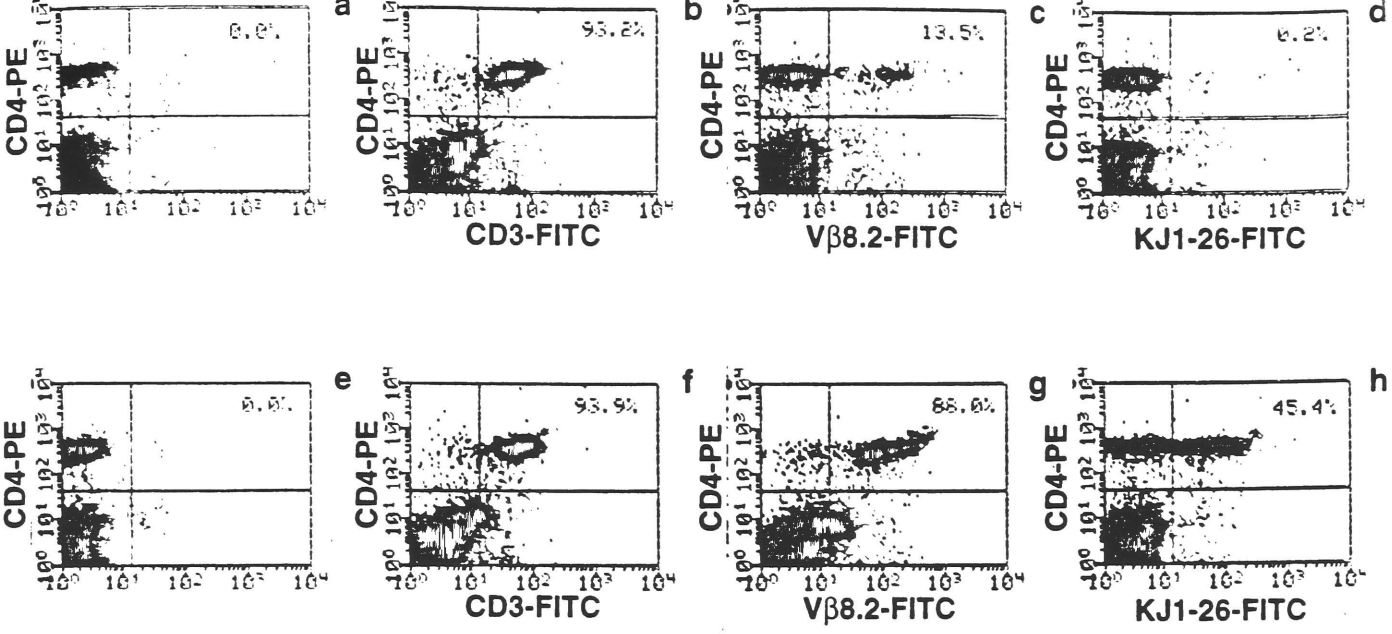


Figure 3 FACS analysis of spleen cells from a transgenic mouse (e-h) and a non transgenic littermate (a-d).

transgenic mice that express both TCR transgenes will react with this mAb; the remaining T cells will be cells that express the transgenic β chain in combination with endogenous α chains. As expected virtually none of the cells in the non transgenic mouse reacted with this mAb whereas 45% of all CD4 cells in the transgenic were KJ1-26$^+$.

Figure 4 shows that the proliferative responses of T cells from the above mice correlated well with their phenotypes. Cells from the transgenic mouse but not from the non transgenic littermate proliferated in response to both native ovalbumin and to the exact peptide epitope OVA 323-339. Cells from both mice responded to SEB, but cells from the transgenic mouse showed an increased response presumably due to the increased number of Vβ8 T cells. As a negative control, the proliferative response of transgenic cells to the superantigen SEA was tested. This toxin reacts with T cells that express Vβ segments other than Vβ8. Figure 4d shows that the nontransgenic T cells responded much better to this superantigen than did transgenic T cells. We also looked at the response to immobilized anti TCR mAbs. Cells from both mice responded well to anti CD3 (Fig. 5a) but only transgenic T cells proliferated in response to the clonotypic mAb (Fig 5b).

I.V. injection of SEB into DO11.10 transgenic mice

Our main goal was to follow the response of a monoclonal population of primary T cells to a superantigen. In initial experiments DO11.1O mice were immunized intravenously with 50 ug each of SEB. 2/6 SEB primed mice died over two experiments. Prior to this no deaths had ever been seen in non transgenic mice treated with identical doses of SEB. The toxic effect is therefore probably due to systemic release of cytokines such as TNF by the transgenic T cells.

Ten days after injection, a significant decrease in the percentage of CD4$^+$ Vβ8$^+$ T cells was seen in SEB primed mice (17.5% in SEB primed mice compared to 32.2% in controls; Fig 6). The ratio of CD4/CD8 Vβ8 T cells was also much lower in SEB primed mice than in controls (Table III). In contrast the percentage of CD4$^+$ KJ1-26$^+$ T cells was only slightly decreased (11.3% in SEB primed mice vs. 13.7% in controls; Fig 6).

Table III. CD4/CD8 ratio of Vβ8$^+$ and KJ1-26$^+$ T cells in control and SEB primed mice

	CD4/CD8: Vβ8$^+$	CD4/CD8: KJ1-26$^+$
Control mice	6.9	10.3
SEB primed mice	2.1	4.2

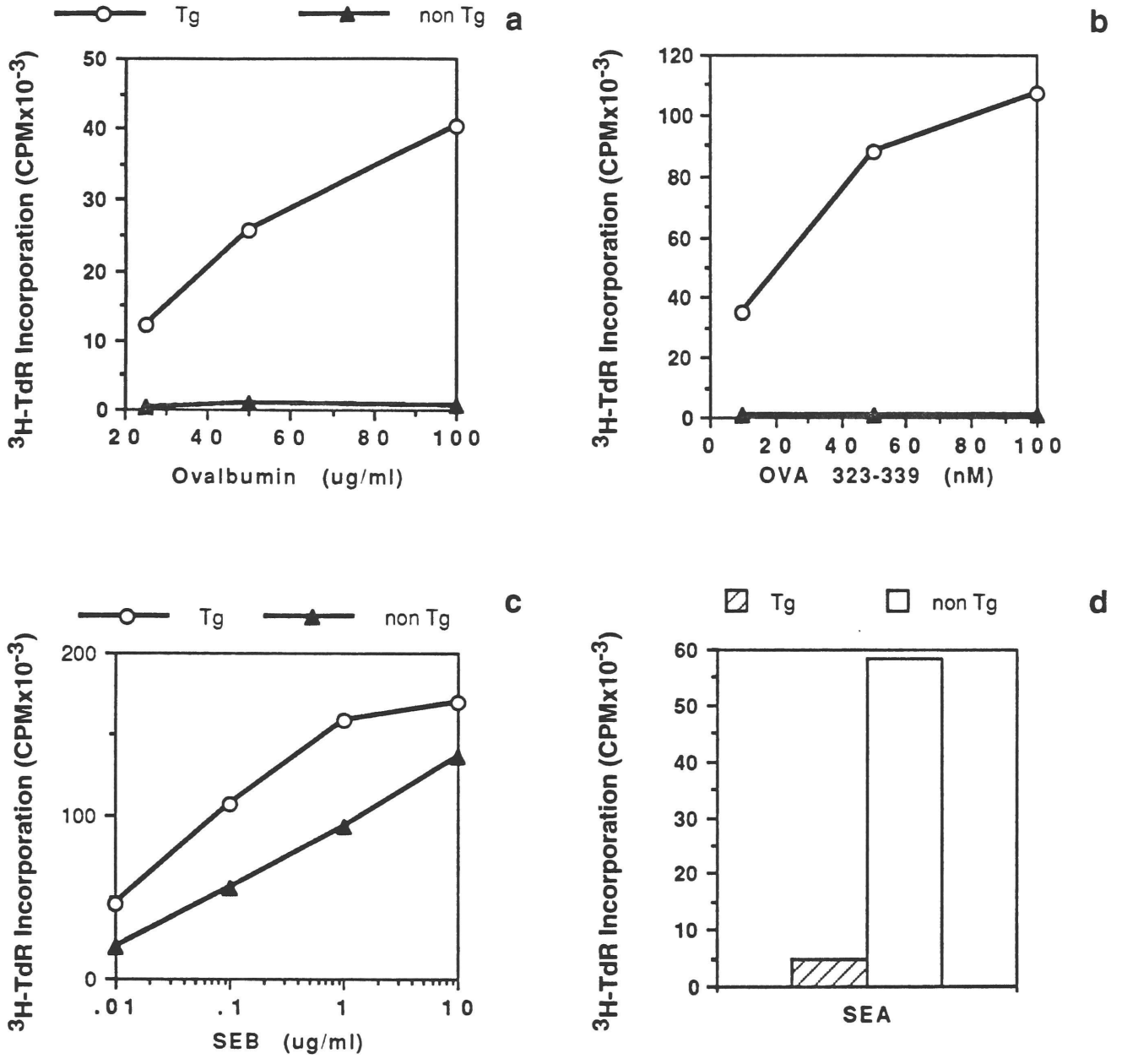


Figure 4. Proliferation of spleen cells from either a transgenic (Tg) mouse (open circles) or a non transgenic littermate (closed triangles) in response to either native ovalbumin (a), OVA 323-339 (b), SEB (c), or 1 ug/ml of SEA (d). Cells were pulsed with ^{3}H thymidine on day 3 and harvested on day 4.

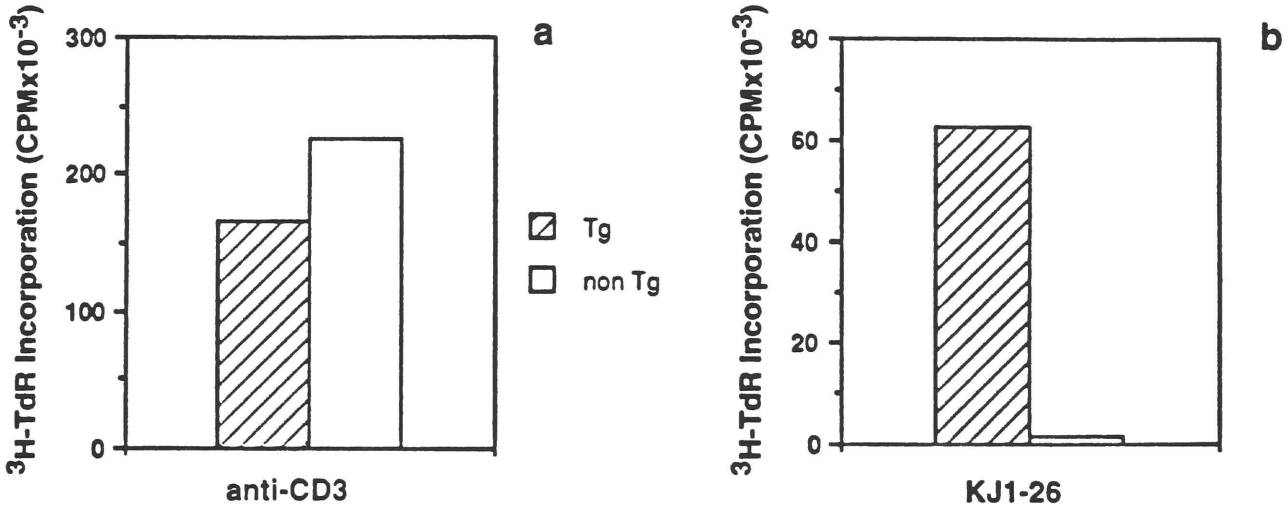


Figure 5. Proliferation of spleen cells from either a transgenic mouse (striped bar) or a non transgenic littermate (open bar) in response to either 2 ug/ml of immobilized anti CD3 (a) or 5 ug/ml of KJ1-26 (b). Cells were pulsed with ^{3}H thymidine on day 2 and harvested on day 3.

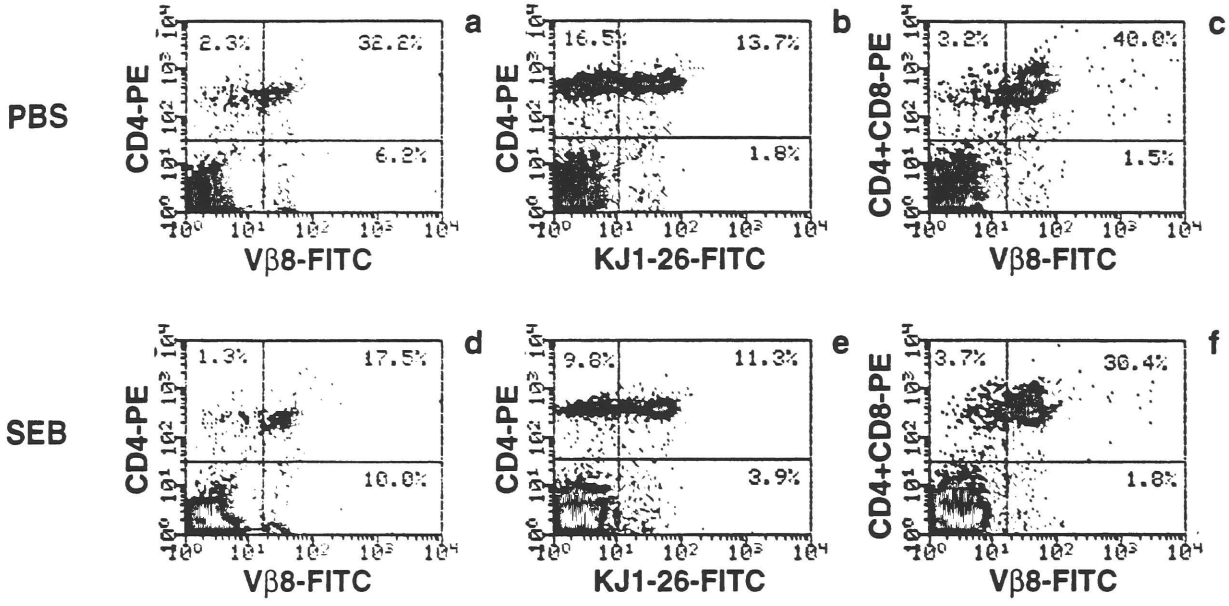


Figure 6 FACS analysis of spleen cells from DO11.10 mice injected intravenously with either PBS (a-c) or SEB (d-f) 10 days earlier.

There was, however, a significant decrease in the ratio of CD4/CD8 KJ1-26$^+$ T cells in SEB primed mice (Table III), implying either a selective deletion of CD4$^+$ cells or a selective expansion of CD8$^+$ T cells. The number of CD4$^-$CD8$^-$ T cells in SEB primed mice was not significantly increased (Fig 6).

Surprisingly, despite the marked decrease in the number of Vβ8 T cells, cells from SEB primed D011.10 mice proliferated as well as control cells in response to both SEB and to OVA 323-339 (Fig. 7). The cells from SEB primed mice however secreted significantly less interleukin 2 (IL-2) in response to SEB, OVA 323-339, and anti CD3 mAb as determined by the decreased proliferation of the IL-2 dependent HT-2 cell line. (Fig. 8).

Subcutaneous injection of SEB and CFA into DO11.10 mice

Because of the high mortality seen in D011.10 mice primed intravenously with SEB, an alternative route of administration, footpad injections with SEB and CFA was used. In contrast to the results seen with intravenous administration, the percentage of Vβ8.2$^+$ and KJ-126$^+$ lymph node (LN) T cells was only slightly reduced in mice primed subcutaneously with SEB compared to controls (Fig.9). There was also no significant increase in the numbers of CD4$^-$CD8$^-$ T cells. In addition, downregulation of CD4, Vβ8, or of the clonotypic receptor was not seen (Fig. 10 and Table IV).

Despite having comparable numbers of KJ-126$^+$ cells, LN cells from SEB primed mice proliferated poorly in response to either SEB or OVA 323-339 (Fig 11. a,b). Reduced proliferation was also seen in response to the lectin Con A and to immobilized anti-CD3 and KJ1-26 mAb (Fig. 12). Purified CD4 cells from tolerized mice had a somewhat improved proliferative response to SEB, to OVA 323-339, and to anti CD3 mAb compared with whole LN cells (Fig. 11,12), but they produced signicantly less IL-2 in response to these stimuli than did control CD4 cells (Table V).

In contrast to this unresponsiveness to specific mitogenic stimuli, CD4 cells from tolerized animals were capable of proliferating in response to PMA and ionomycin (Fig.

Table IV.

Mean channel fluorescence of CD4, Vβ8 and KJ1-26 on LN T cells from mice primed with either CFA + PBS or CFA + SEB.

	Treatment of mice	
	CFA + PBS	CFA + SEB
CD4 expression on KJ1-26 cells	347	322
CD4 expression on Vβ8 cells	339	295
KJ1-26 expression on CD4 cells	81	72
Vβ8 expression on CD4 cells	68	67

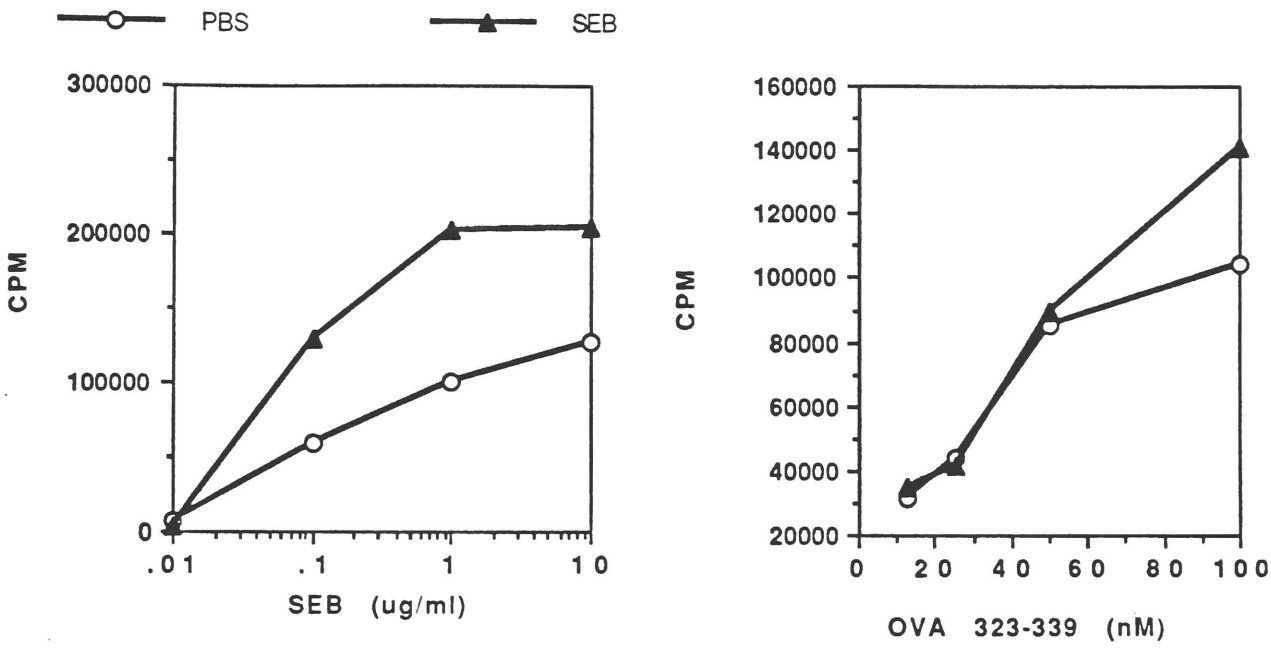


Figure 7 Proliferation of spleen cells from either PBS (open circles) or SEB (closed triangles) primed DO11.10 mice in response to either SEB (a) or OVA 323-339 (b). Cells were pulsed with ^{3}H thymidine on day 3 and harvested on day 4.

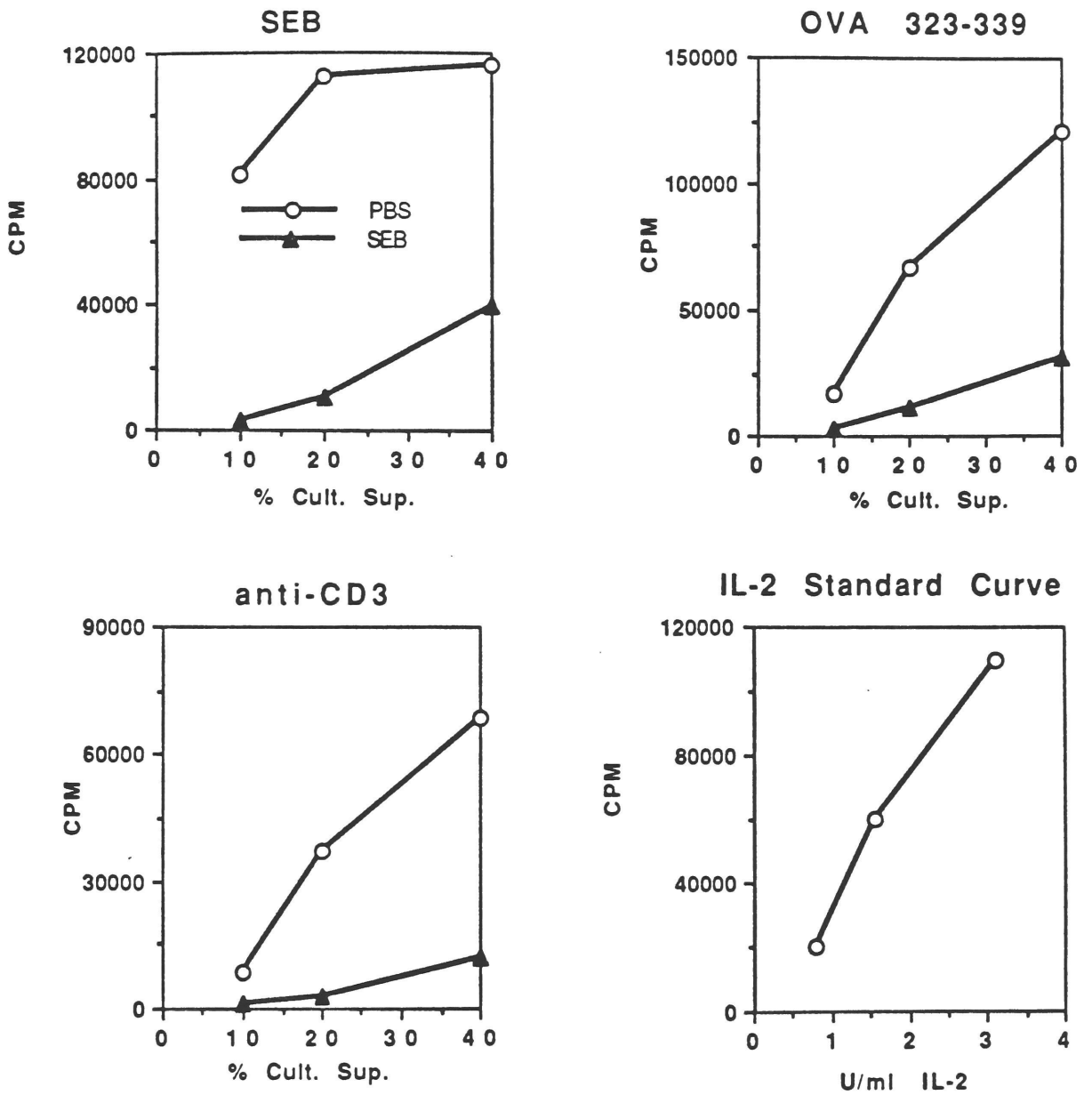


Figure 8 Proliferation of HT-2 cells in response to culture supernatant from spleen cells from either PBS (open circles) or SEB (closed triangles) primed DO11.10 mice that had been stimulated with either 10 ug/ml of SEB, 100 nM of OVA 323-339, or 2 ug/ml of anti CD3. Some cells were grown with different concentrations ofrecombinant mouse IL-2 and a standard curve generated. Cells were pulsed with ^{3}H thymidine after 18 hrs and harvested 12 hrs later.

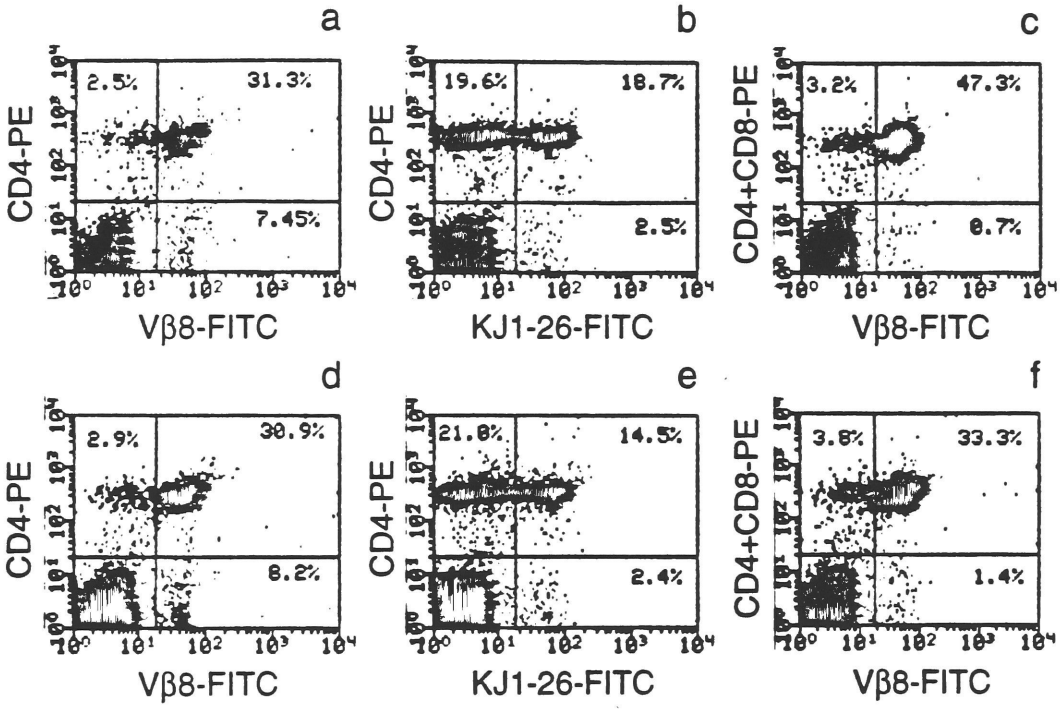


Figure 9 FACS analysis of lymph node (LN) cells from DO11.10 mice primed with CFA + PBS (a-c) or CFA + SEB (d-f) 10 days earlier.

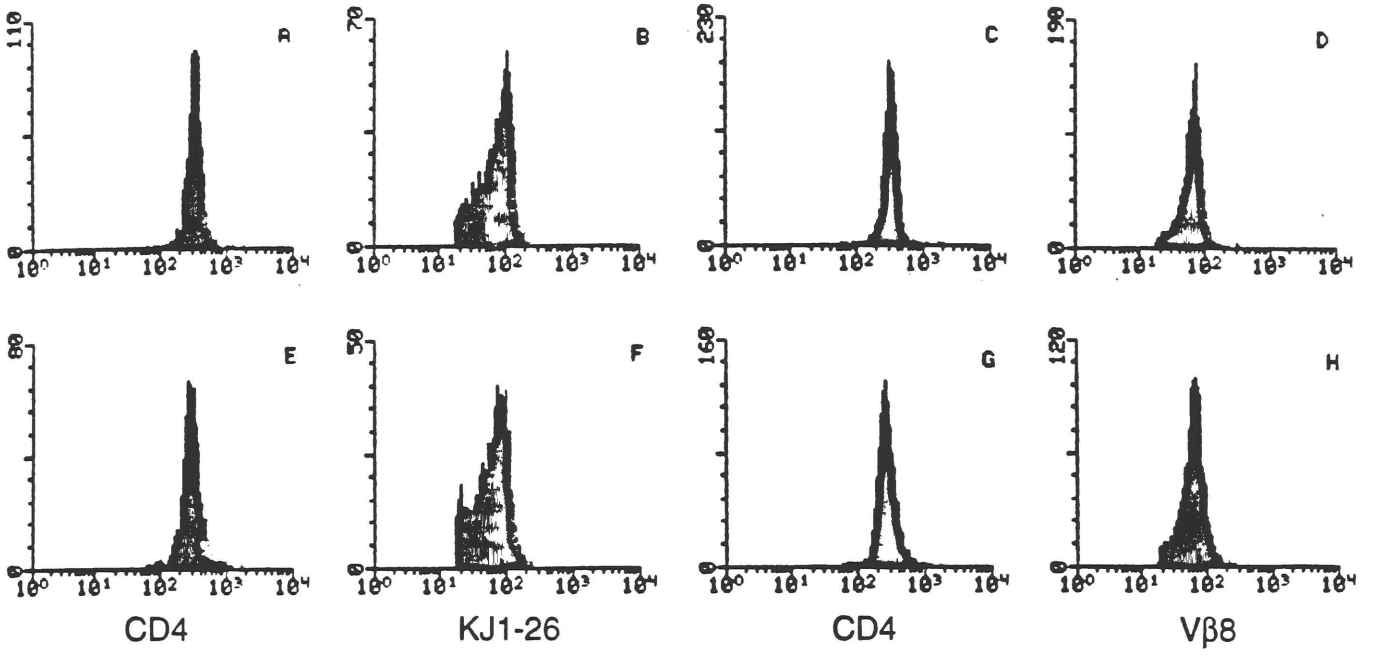


Figure 10 Surface markers on T cells from control (panels A-D) or SEB primed DO11.10 mice (panels E - H). Cells were double stained with CD4 and Vβ8 or CD4 and KJ1-26. Histograms show the expression of the specified antigen on double positive cells.

A,E CD4 expression on KJ1-26$^+$ cells

B,F KJ1-26 expression on CD4$^+$ cells

C,G CD4 expression on Vβ8$^+$ T cells

D,H Vβ8 expression on CD4$^+$ T cells

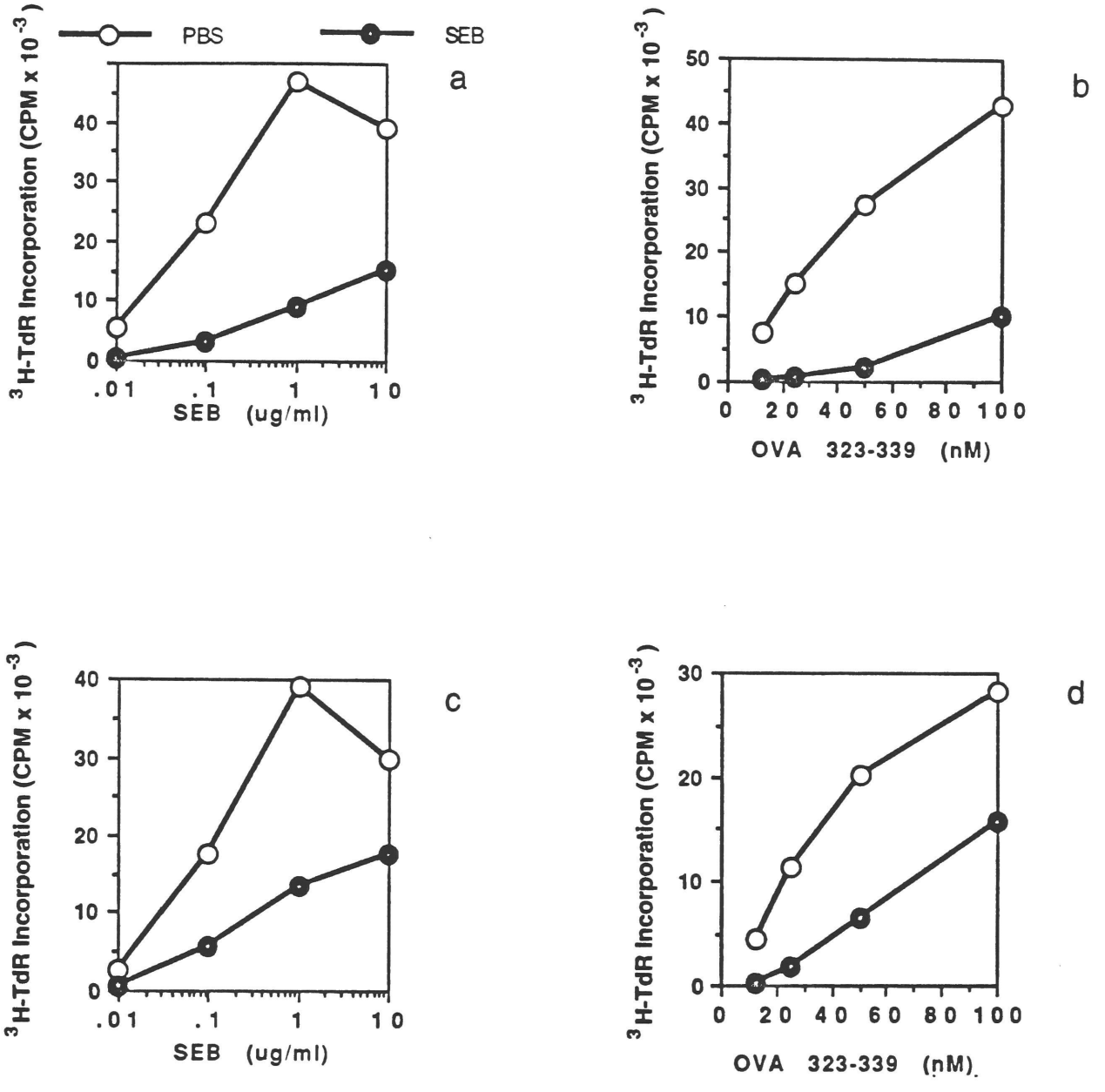


Figure 11 Proliferation of LN T cells (a,b) or purified CD4$^+$ cells (c,d) in response to SEB (a,c,) or OVA 323-339 (b,d). Cells were pulsed with ^{3}H thymidine on day 3 and harvested 16 hrs later.

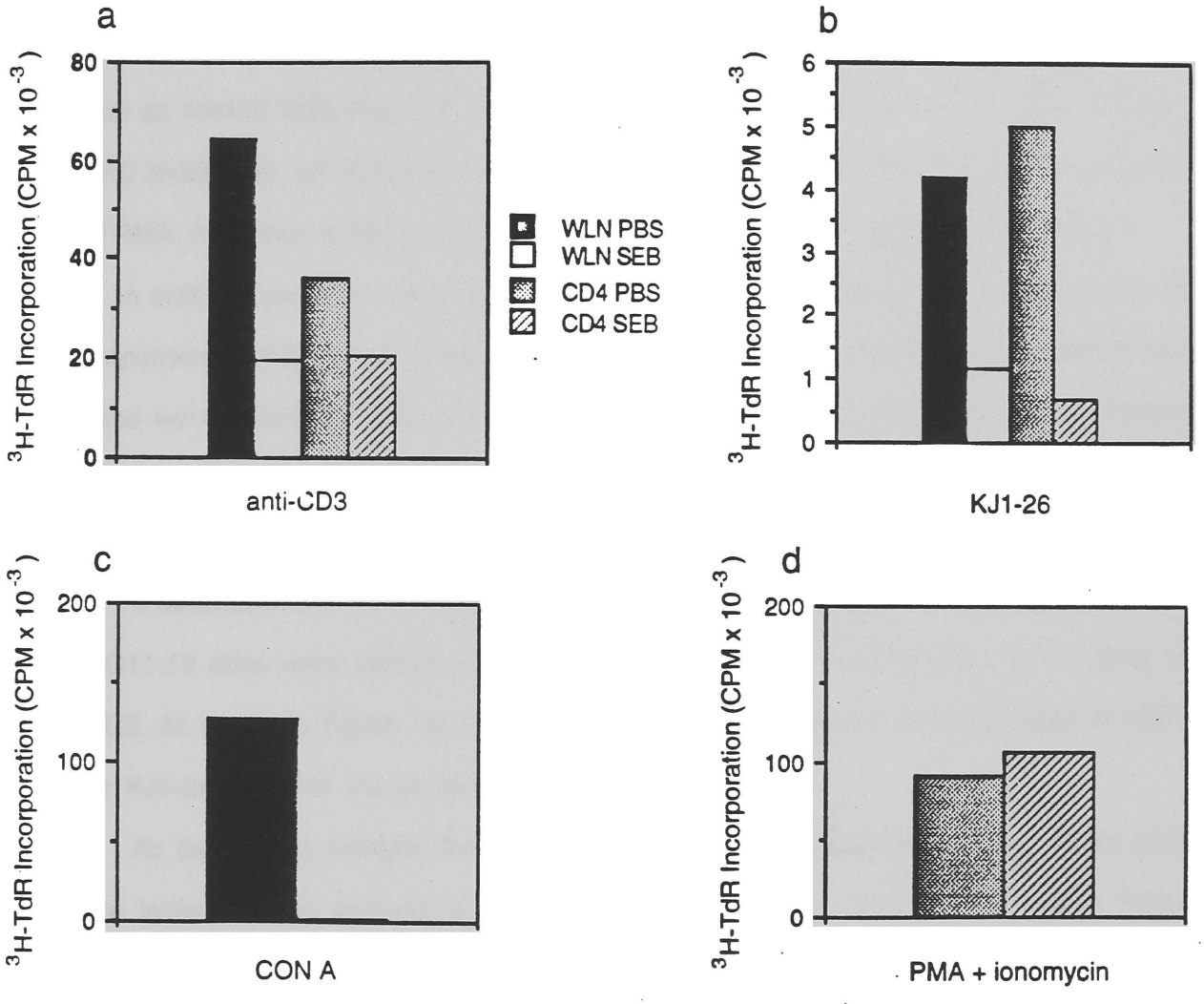


Figure 12 Proliferative response of whole lymph node (WLN) or CD4 cells from either PBS (control) or SEB primed mice to a, CD3 (2 ug/ml) b, KJ1-26 (2.5 ug/ml) c, Con A (2.5 ug/ml) or d, PMA (10 ng/ml) + ionomycin (200 ng/ml). Cells stimulated with Mab were pulsed on day 2 and harvested14 hrs later. Cells from other experiments were pulsed on day 3 and harvested on day 4.

12d), suggesting that a proximal signal transduction step was affected in anergic cells. In order to determine whether calcium mobilization was the defective step, we compared the response of control and anergic cells to Con A. In marked contrast to their inability to proliferate to this mitogen, LN cells from SEB primed mice could mobilize calcium as well as control cells (Fig. 13). To determine whether the defect was due to decreased PKC production, we stimulated the cells with SEB or with OVA 323-339 in the presence of PMA. As shown in Figure 14, PMA was unable to reverse anergy to either antigen.

In order to determine whether the apparent tolerance could be due to suppression of responsiveness by a cell subpopulation in SEB primed mice, LN cells from SEB primed mice were cocultured with control LN cells. The presence of tolerant cells in coculture did not affect the response of control cells to either SEB or OVA 323-339 (Figure 15).

Subcutaneous injection of OVA 323-339 and CFA into DO11.10 mice

To determine whether this tolerance was the normal outcome of an immune response, DO11.10 mice were primed with 50 ug of OVA 322-339 or with either 25 or 50ug of SEB. As shown in Figure 16, there were no major differences in the percentage of $V\beta8^+$ or KJ1-26^+ cells in any of these groups.

As the homing receptor CD44 is thought to be upregulated on memory T cells (92), we looked at the expression of this molecule on $V\beta8^+$ and KJ1-26^+ cells. CD44 expression was markedly increased on KJ1-26^+ LN cells from OVA primed mice (Fig 17A and Table V). In contrast there was only a moderate increase in CD44 expression on KJ1-26^+ cells from mice primed with 50 ug of SEB (Fig 17B and Table VI). The expression of CD44 on the entire Vβ8 population was only slightly increased in LN cells from mice treated with SEB, and remained unchanged in OVA primed mice (Fig. 17 C,D and Table VI).

Cells from mice primed with OVA proliferated normally (Fig 18a) and secreted significant levels of IL-2 (Table VII) in response to SEB. In contrast their proliferative response to OVA 323-339 was greatly increased (Fig 18b). A dose response effect of SEB is seen in the proliferative response to both SEB and OVA 323-339.

Table V. IL-2 secretion (U/ml) in response to various stimuli by purified CD4 cells from SEB primed and PBS primed (control) DO11.10 mice.

	SEB (10 ug/ml)	OVA 323-339 (100 nm)	anti CD3 (2 ug/ml)
Control	67.8	23.6	6.81
SEB primed	3.3	4.4	0.73

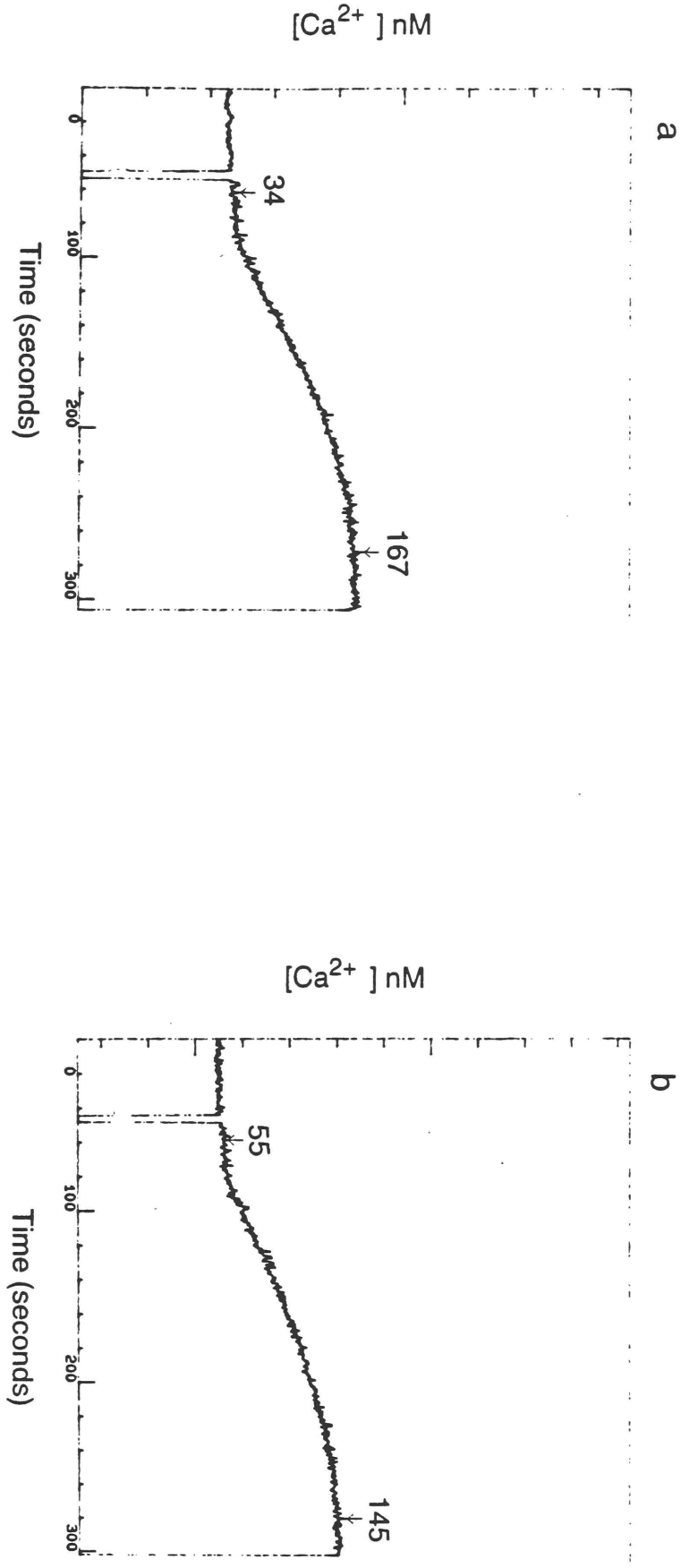
Table VI. Mean channel fluorescence of CD44 on Vβ8 and KJ126 LN T cells from mice primed with either CFA + PBS (control), CFA + 50 ug of OVA 323-339 (OVA) or CFA + 50 ug of SEB (SEB)

	Control	OVA	SEB
CD44 expression on Vβ8 cells	139	137	181
CD44 expression on KJ1-26 cells	168	275	235

Table VII. IL-2 secretion (U/ml) in response to various stimuli by LN cells from DO11.10 mice primed with either CFA only (control), 25 ug SEB (SEB 25), 50 ug SEB (SEB 50), or 50 ug OVA 323-339 (OVA).

	Treatment of LN cells		
Treatment of mice	SEB (10 ug/ml)	OVA 323-339 (100 nm)	anti CD3 (2 ug/ml)
CFA only	110	11.4	6.7
SEB 25	2.4	1.0	< 0.4
SEB 50	3.5	0.9	< 0.4
OVA	50	23.2	2.9

Figure 13 Calcium flux assay. Indo 1 loaded control (a) or anergic (b) cells were stimulated with 10 ug/ml of Con A. The break in the graph represents the point of Con A addition. The numbers are $[Ca^{2+}]_i$ concentration at the indicated points.


a
[Ca2+] nM
Time (seconds)
0
100
200
300
34
167
b
[Ca2+] nM
Time (seconds)
0
100
200
300
55
145

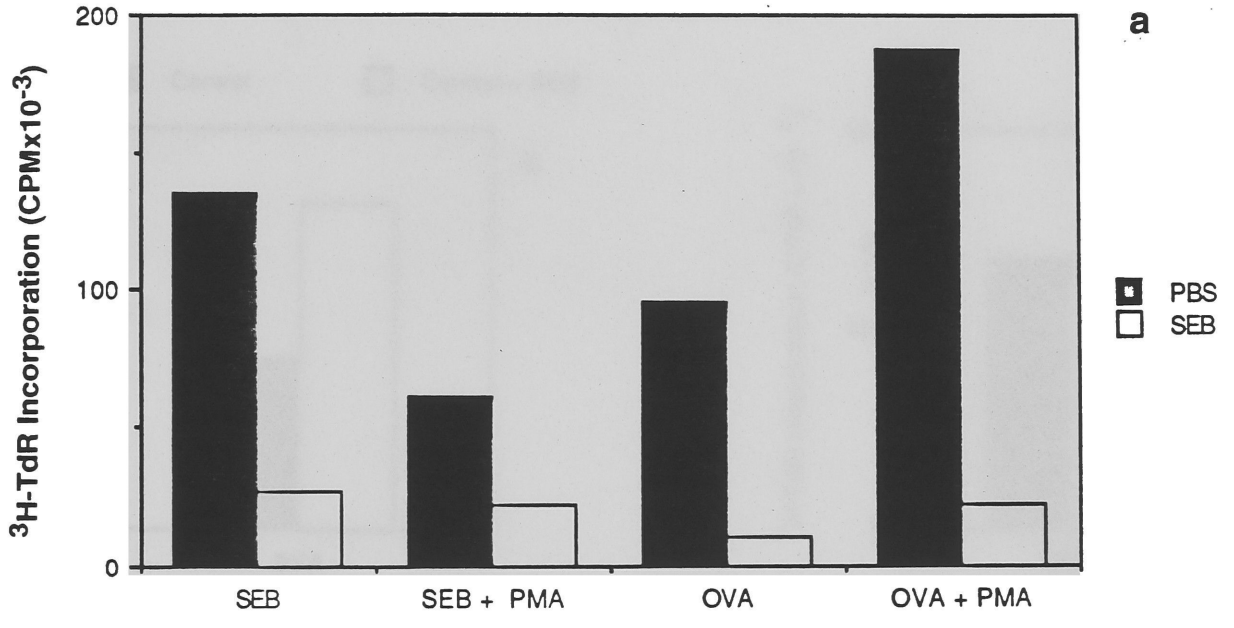


Figure 14 Proliferative response of lymph node (LN) cells from either PBS (control) or SEB primed mice to 1 ug/ml of SEB or 100nM of OVA 323-339 in the presence or absence of 10 ng/ml of PMA. Cells were pulsed on day 3 and harvested on day 4.

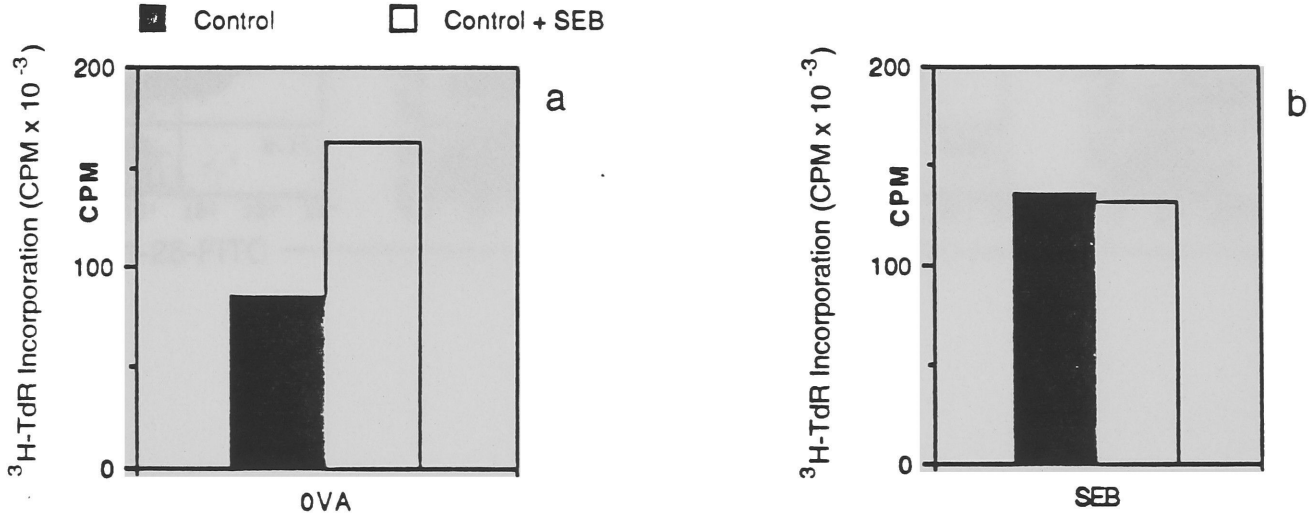


Figure 15 Coculture of LN T cells from control and SEB primed mice. Control cells (filled bar) or control cells + anergic cells (open bar) were cultured in the presence of 100 nM OVA 323-339 (a) or 1 ug/ml of SEB (b). 2 X10^5 cells from each group were cocultured. Cells were pulsed on day 3 and harvested on day 4.

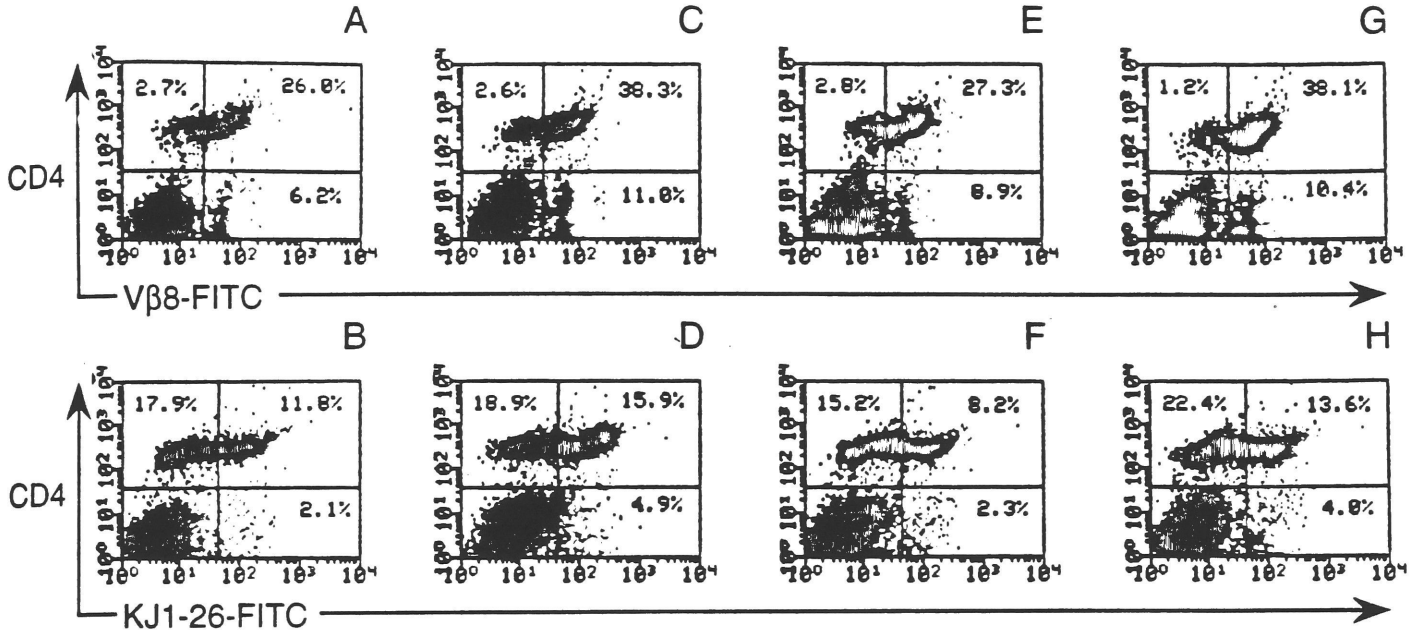


Figure 16 FACS analysis of LN cells from DO11.10 mice primed with either CFA + PBS (A,B) CFA + 50 ug of OVA 323-339 (C,D), CFA + 50 ug of SEB (E,F) or CFA + 25 ug of SEB (G,H)

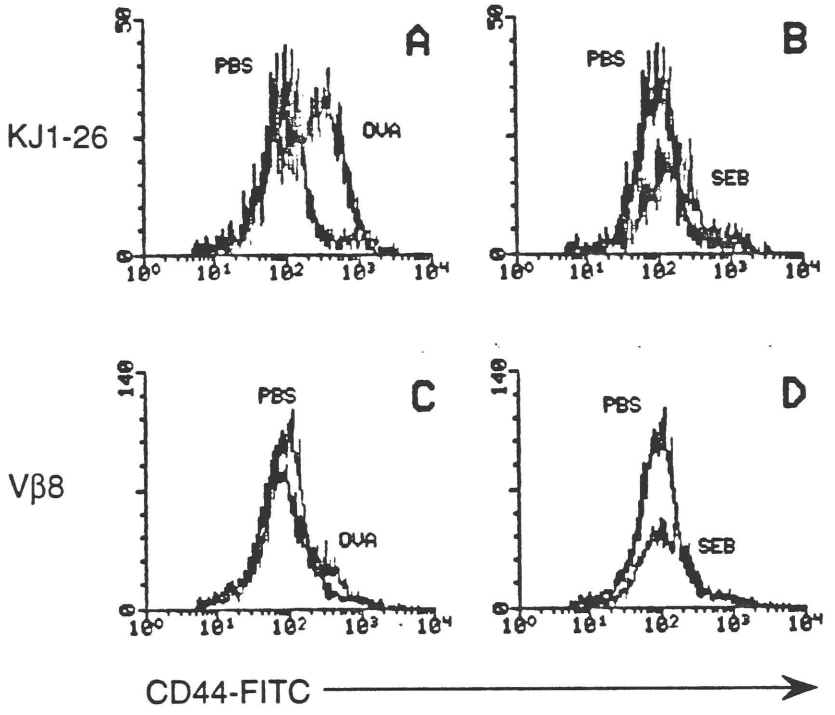


Figure 17 Expression of CD44 on DO11.10 LN T cells. Cells were double stained with either CD44 and KJ1-26 or CD44 and Vβ8. KJ1-26$^+$ (A,B) or Vβ8$^+$ (C,D) cells were gated on and their levels of CD44 displayed as histograms. Overlays are shown of histograms from cells from CFA + PBS primed mice (PBS) vs. cells from either CFA + 50 ug SEB (SEB) or CFA + OVA 323-339 (OVA) primed mice.

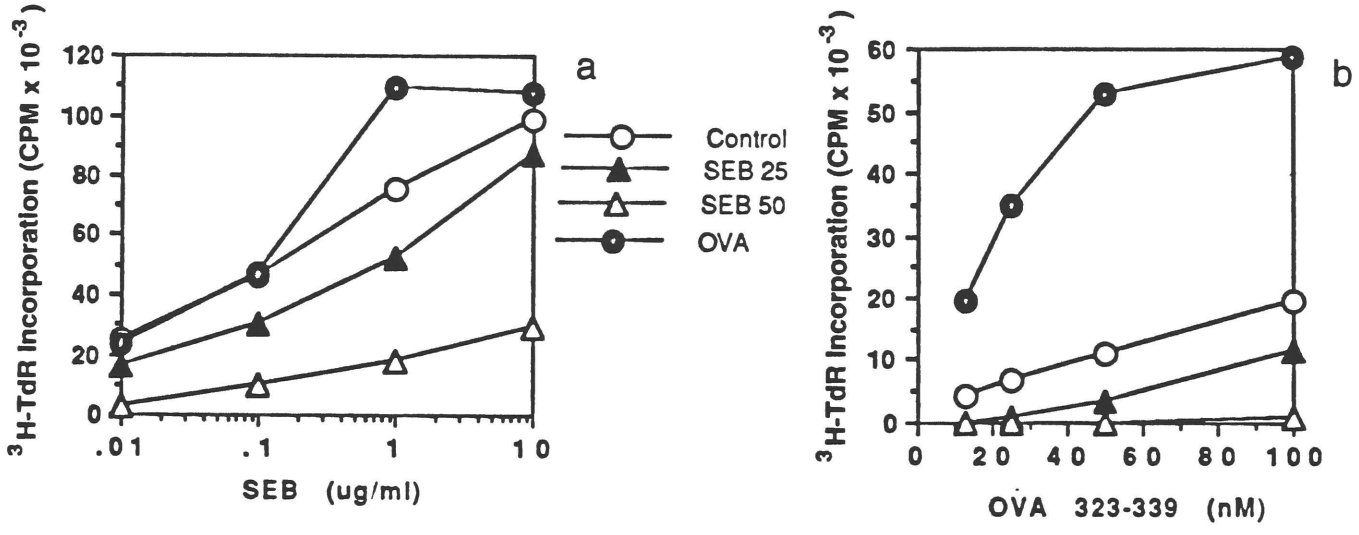


Figure 18 Proliferative response of cells from DO11.10 mice primed with either CFA + PBS (Control), CFA + 25 ug of SEB (SEB-25), CFA + 50 ug of SEB (SEB-50), or CFA + 50 ug of OVA 323-339 (OVA) in response to either SEB (a) or OVA 323-339 (b). Cells were pulsed after 48 hrs and harvested 14 hrs later.

Cells from mice treated with 25 ug of the superantigen responded better than mice treated with 50ug (Fig 18a,b). However cells from both sets of mice secreted very low levels of IL-2 in response to either of the two antigens (Table VII). Similar results were obtained in three separate experiments as shown in Table VIII.

Addition of exogenous IL-2

Since IL-2 secretion was markedly reduced in anergic cells, and since IL-2 is necessary for T cell proliferation, the effect of adding exogenous IL-2 on anergy was tested. Mice were primed with either 25ug or 50 ug of SEB. No deletion of Vβ8 or KJ1-26 cells (Fig. 19) was seen. In contrast to earlier experiment, there was no difference in the proliferative response of cells from mice treated with 25 ug of SEB and those treated with 50 ug of SEB (Fig. 20 a,b, Fig. 21a). 100 U/ml of IL-2 did not reverse anergy to either SEB (Fig. 20a), OVA 323-339 (Fig 20b), or to immobilized anti TCR mAbs (Fig. 21b).

Table VIII. Proliferative responses of lymph node cells from DO11.10 mice primed with either 25 ug SEB (SEB 25), 50 ug SEB (SEB 50), or 50 ug OVA 323-339 (OVA). Stimuli are 1 ug/ml of SEB, 50 nM of OVA 323-339, 2 ug/ml of immobilized anti CD3 or 5 ug/ml of KJ126 mAb, 100 ug/ml of ovalbumin. Results are shown as % of the proliferative response of cells from DO11.10 mice primed with CFA only as a control. Thymidine incorporation was measured as described in Materials and Methods. ND: not determined.

Treatment of mice		SEB	OVA	αCD3	KJ1-26	Ovalbumin
Expt.1	SEB 50	20.9%	14.3%	33.9%	26.2%	ND
	OVA	89.6%	137%	122%	127%	ND
Expt.2	SEB 50	48.5%	33.4%	37.7%	34.4%	38.6%
	OVA	112%	310%	79.2%	122%	350%
Expt.3	SEB 50	22.9%	0%	3.2%	ND	9.1%
	SEB 25	69.1%	31%	8.7%	ND	ND
	OVA	143%	470%	64.3%	ND	189%

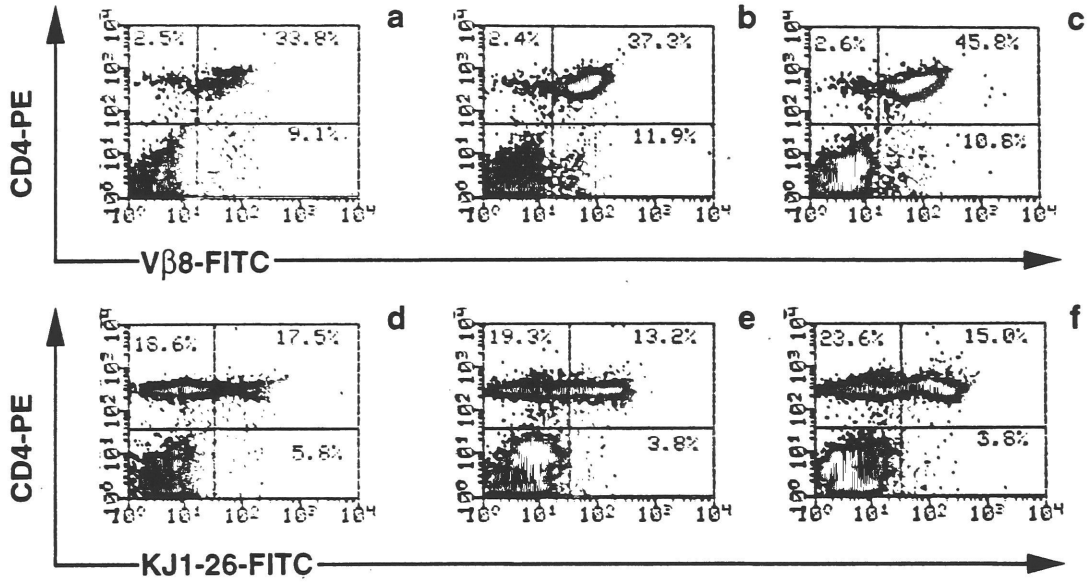


Figure 19 FACS analysis of LN cells from DO11.10 mice primed with either CFA + PBS (a,d), CFA + 50 ug of SEB (b,e), or CFA + 25 ug of SEB (c,f).

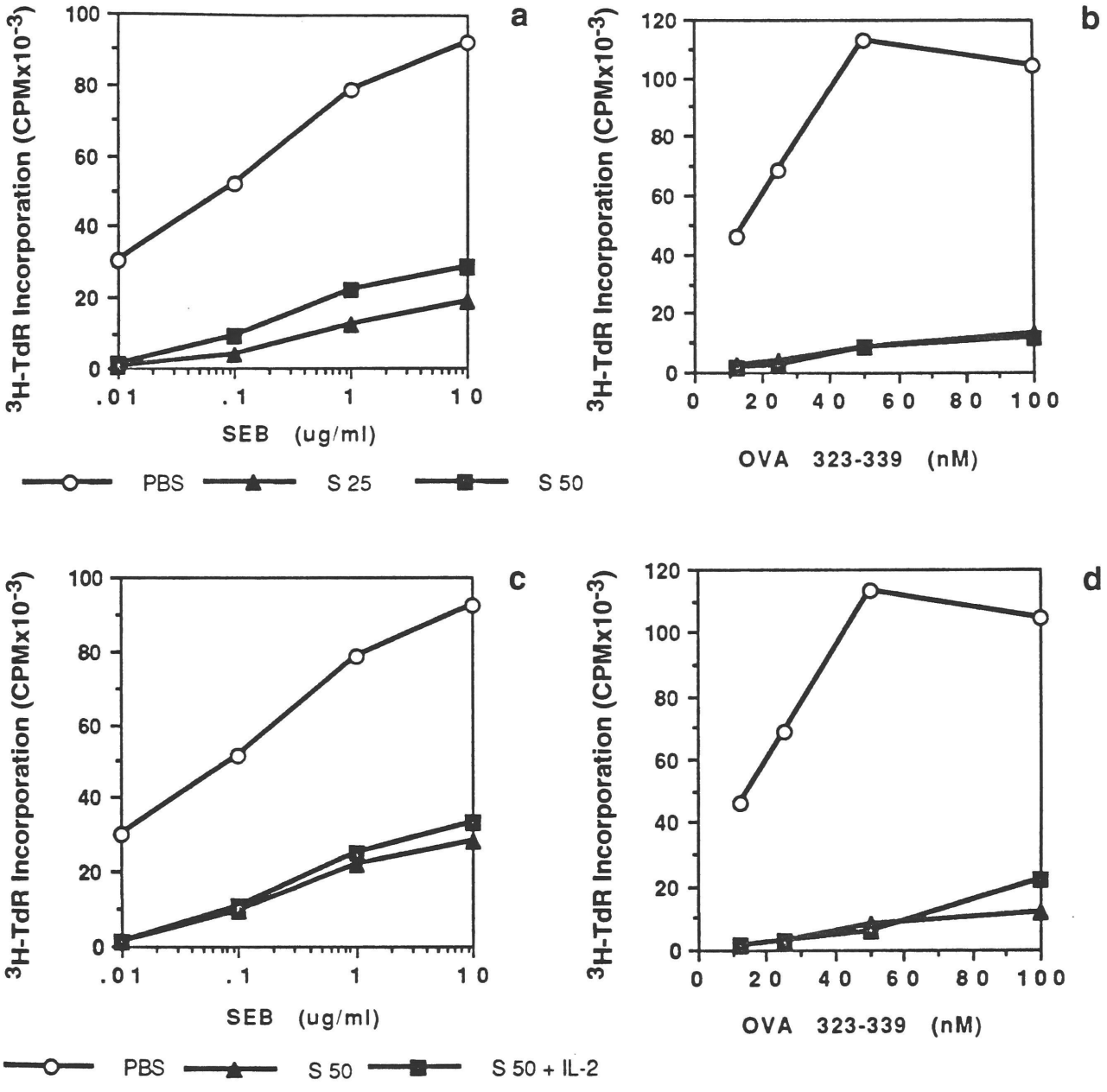


Figure 20 Proliferative response of cells from mice primed with either CFA + PBS (PBS), CFA + 25 ug of SEB (S-25), or CFA + 50 ug of SEB (S-50) in response to either SEB (a) or OVA 323-339 (b). In c and d 100 U/ml of recombinant mouse IL-2 was added to each dose of mitogen. This dose of IL-2 had very little effect on control cells (not shown). Cells were pulsed after 48 hrs and harvested 14 hrs later.

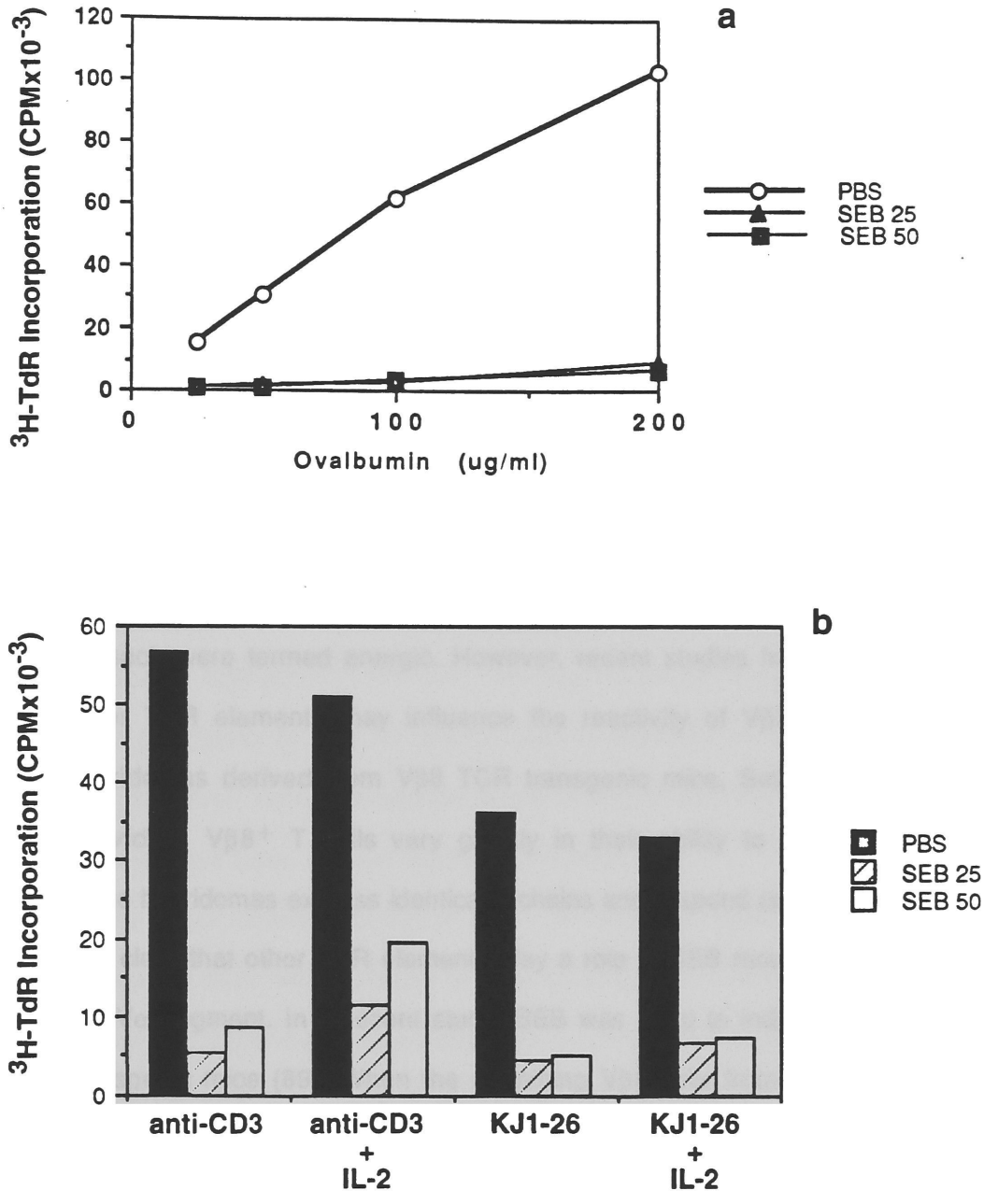


Figure 21 Proliferative response of cells from mice primed with either CFA + PBS (PBS), CFA + 25 ug of SEB (S-25), or CFA + 50 ug of SEB (S-50) in response to either native ovalbumin (a) or to immobilized anti CD3 or KJ1-26 in the presence or absence of 100 U/ml of recombinant mouse IL-2 (b). Cells treated with mAb were pulsed after 48 hrs and harvested 14 hrs later, Cells stimulated with native ovalbumin were pulsed on day 3 and harvested on day 4.

Chapter 1.4 (Discussion)

Superantigens stimulate T cells primarily through the Vβ element of the T cell receptor (TCR). The development of mAbs to different Vβ elements has enabled investigators to physically identify superantigen reactive T cells, with major implications for the study of tolerance.

White et al. first showed that treating neonatal mice with the superantigen SEB resulted in the selective deletion of immature thymocytes expressing SEB reactive receptors (58). This work was followed up by studies showing that peripheral T cells in adult mice could also be tolerized by SEB (70,71). Inoculating mice with SEB results in clonal expansion followed by partial deletion of $V\beta8^+$ T cells (72,73). The remaining cells were hyporesponsive to SEB (70,71), and to anti Vβ8 mAbs (71) and as such were termed anergic. However, recent studies have shown conclusively that other TCR elements may influence the reactivity of Vβ8 to SEB. Using a panel of hybridomas derived from Vβ8 TCR transgenic mice, Smith et al. demonstrated that individual $V\beta8^+$ T cells vary greatly in their ability to respond to SEB (6). Since these hybridomas express identical β chains and respond comparably to anti TCR mAbs, it is clear that other TCR elements play a role in SEB recognition. A likely candidate is the Vα segment. In a recent study, SEB was used to induce clonal deletion in Vβ8.1 transgenic mice (89). When the remaining Vβ8 cells from these mice were compared to Vβ8 cells from control transgenic mice, a marked skewing of the Vα repertoire was seen (89).

In addition, in a recent study where Vβ8.2 TCR transgenic mice were tolerized with SEB, it was shown that while the remaining T cells did not respond to SEB, they proliferated as well as control cells in response to anti TCR mAbs (94).

Taken together, these studies suggest a possible alternative explanation to anergy, namely that priming mice with SEB results in the selective deletion of highly reactive

clones and that this selective deletion, rather than true clonal anergy, may account for some of the diminished response to SEB.

We used αβ TCR transgenic mice to address this issue. T cells from these mice express both TCR chains from the OVA specific hybridoma DO11.10, respond to OVA 323-339 in the context of I-A^d, and can be detected by the clonotypic mAb KJ1-26. In addition the β chain is Vβ8 and the cells are thus specific for the superantigen SEB as well as for the conventional antigen OVA. We reasoned that KJ-126$^+$ cells expressed identical TCR elements and as such should all be equally responsive to SEB. Thus we would be able to follow a monoclonal response to a superantigen and determine whether true clonal anergy was occurring.

When DO11.10 mice were immunized intravenously with SEB, partial clonal deletion of Vβ8 and KJ1-26 CD4 cells was observed. Surprisingly, the remaining cells could proliferate as well as control cells in response to SEB and OVA 323-339. The cells, however, secreted reduced levels of IL-2 in response to these stimuli suggesting that they had been at least partially tolerized.

No major deletion of either Vβ8$^+$ or KJ1-26$^+$ cells was seen when DO11.10 transgenic mice were primed subcutaneously with SEB emulsified in CFA, yet cells from these mice were hyporesponsive to OVA 323-339 as well as to SEB, a finding consistent with true clonal anergy. It is not clear why different results were obtained with the different routes of inoculation. It is possible that different antigen presenting cells were involved in the two systems leading to different outcomes. It is also possible that the emulsification of SEB in CFA led to a slower release of the antigen and that this difference in kinetics resulted in anergy and not deletion. The response of mice treated intravenously with SEB is similar to that seen when Vβ8.1 TCR transgenic mice are tolerized with Mls-1a spleen cells. T cells from these mice will proliferate but not secrete IL-2 in response to Mls antigen present on activated B cells (101).

While T cells from mice primed subcutaneously with SEB appeared to be anergic, we cannot completely rule out selective deletion as being responsible for some of the observed tolerance to SEB, as not all Vβ8.2$^+$ T cells express the transgenic α chain (these cells are KJ1-26$^-$). It is thus possible that a small number of highly SEB reactive Vβ8$^+$ KJ1-26$^-$ T cells were deleted. While this might explain why the IL-2 response was more affected in response to SEB than to OVA, it would not explain the diminished response to OVA under the same conditions. For this, clonal anergy would appear to be the only explanation: only KJ1-26$^+$ cells can respond to OVA and no deletion of these cells was seen in mice primed with 25 ug of SEB. Another possible explanation is downregulation of TCR and/or CD4 in SEB primed mice, leading to anergy. However, no significant down regulation of either Vβ8, the clonotypic TCR, or CD4 was seen in cells from SEB primed mice. Finally, active suppression is not likely to be the cause of the observed tolerance as cells from SEB primed mice did not inhibit the response of control cells to either SEB or to OVA 322-339.

This study confirms and extends recent work by Perkins et al., in which Vβ8.2 TCR transgenic mice were inoculated with SEB (95). T cells from these mice were non responsive to different superantigens, mitogens, and anti CD3 mAbs. By using αβ TCR transgenic mice, we have been able to follow a monoclonal response and have thus been better able to rule out selective deletion of highly SEB reactive Vβ8 clones as a cause of tolerance. Further supporting the interpretation that anergy occurred, we have shown that IL-2 production as well as proliferation is affected in the anergic cells, noteworthy because the inability to secrete IL-2 has been reported as the major defect present in anergic T cell clones (96).

Defect in anergic T cells

It is noteworthy that the cells were anergic to both a conventional antigen and a superantigen, as several studies have implied that the two sets of antigens are coupled to different signalling pathways (97-100). If this is true, then our results would

imply that the defect in anergic cells lies in a step common to both pathways. The ability of PMA and ionomycin to stimulate anergic cells suggests that a proximal signal transduction step is affected, but this step is not likely to be calcium mobilization as anergic cells were able to mobilize calcium normally in response to Con A. Similar results have been recently reported by Yui et al. in Vβ8.1 TCR transgenic mice tolerized with spleen cells fom mice bearing the endogenous superantigen Mls-1. In their system, T cells were able to mobilize calcium but not proliferate in response to cross linking with anti TCR mAbs (101). In the same system, diminished tyrosine phosphorylation of two unidentified protein substrates in response to anti-CD3 stimulation was demonstrated (102). The characterization of these proteins will probably lead to a better understanding of the exact molecular defect present in anergic primary T cells.

It is interesting that the addition of IL-2 did not reverse anergy in our system. This would imply that decreased IL-2 secretion alone is not the cause of the poor proliferative response of the cells to various antigens. This again suggests that a proximal rather than a distal signal transduction step is affected. Similar results were found by Perkins et al. when Vβ8.2 TCR transgenic mice were tolerized with SEB. In another system, in which Vβ8.1 TCR transgenic mice were tolerized with Mls-1a spleen cells, it was found that IL-2 did not restore the proliferative response of CD4 T cells to either Mls-1a stimulator cells or to anti CD3 mAb (102). The response of CD8 T cells to anti-CD3 could, however, be restored by the addition of exogenous IL-2 (102). In a more recent study where Vβ 8.2 TCR transgenic mice were tolerized with SEB, it was found that 30 U/ml of exogenous IL-2 would fully restore the proliferative response to SEB (94). It is not clear why different results are seen in different systems, but it is possible that the difference in the genetic backgrounds of these mice as well as the difference in the transgenic Vβ elements expressed may affect the final outcome.

Anergy in murine T cell clones: I. Lack of costimulation

T cell clonal anergy has been best described and characterized in work done with murine IL-2 secreting (Th-1) T cell clones by Ronald Schwartz and his colleagues. In initial experiments, Jenkins and Schwartz demonstrated that chemically fixing antigen presenting cells with either paraformaldahyde or 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide (ECDI), and then using these cells to present peptide to T cell clones, would result in a long lasting state of anergy (103). It was later found that the addition of unfixed allogeneic antigen presenting cells (which themseves could not present the peptide) to the fixed syngeneic cells and peptide would induce proliferation and not anergy in the T cell clones (104). This work suggested that the fixed cells could present the peptide, but that another essential signal (present on the unfixed allogeneic cells) was destroyed by fixation.

In a more physiological model, Quill and Schwartz demonstrated that planar membranes pulsed with peptide would also induce long term anergy in T cell clones (44). This further suggested that a second or costimulatory signal was required to induce proliferation in T cell clones. The planar membrane plus peptide was able to induce partial activation of the cloned T cells as determined by an increase in cell volume and interleukin-3 (but not IL-2) production. Factors that blocked this partial activation, such as mAb to MHC class II molecules, also blocked the subsequent anergy.

More recently it has been shown that anergy can also be induced by treating T cell clones with either Con A (105) or anti-CD3 mAb (106) in the absence of accessory spleen cells. Thus it appears that the engagement of the T cell receptor in the absence of a costimulatory signal may lead to anergy. This costimulatory signal has recently been determined to be a CD28 ligand as antibodies to this molecule prevent the induction of anergy in T cell clones treated with fixed antigen presenting cells and peptide (107).

Mechanism of anergy induction in T cell clones

Decreased IL-2 production appears to be the major defect in anergic T cell clones; the addition of exogenous IL-2 reverses anergy (108) although it does not prevent its induction (44). Experiments with cycloheximide have suggested that protein synthesis is required for anergy induction (44). A rise in intracellular calcium levels also appears to be essential as the calcium chelator EGTA blocks anergy induction (109), ionomycin by itself can anergize T cell clones (109), and anergy can be prevented by cyclosporin A (110), a drug that, among other things inhibits the calcium/calmodulin/calcineurin pathway (81).

The exact molecular defect present in anergic cells has not been determined although some progress has been made. Engagement of the T cell receptor normally results in the activation of several tyrosine kinases including $p56^{lck}$ and $p59^{fyn}$. The enzyme phospholipase C is activated by this tyrosine phosphorylation and this results in the hydrolysis of phosphatidyl inositol bisphosphate into 1,4,5 trisphosphate, which produces an increase in intracellular calcium, and diacylglycerol which activates protein kinase C. These two signals then activate several factors that initiate transcription of the IL-2 gene. However, additional signalling through the CD28 coreceptor is required for optimal IL-2 production.

Some studies suggest that anergized T cell clones can mobilize calcium normally in response to T cell receptor ligation (111), but other investigators have found anergic cells to be defective in calcium flux and phosphatidylinositol hydrolysis (112). Recently it has been shown that anergized T cell clones express decreased levels of $p56^{lck}$ (113) and increased levels of $p59^{fyn}$ (113,112) in response to stimulation. Interestingly, when anergy is reversed with IL-2, the clones express normal levels of both kinases (113). Furthermore a decrease in the tyrosine phosphorylation levels of two uncharacterized proteins, p38 and p71 is seen (114). This is particularly interesting as the same pattern is seen in T cells from Vβ8.1 TCR transgenic mice

tolerized in vivo with Mls-1a spleen cells (102). This might suggest that the types of anergy have similar mechanisms.

Go and Miller have looked at the at the pattern of transcription factors associated with the IL-2 promoter in untreated and anergic T cell clones. They found that during the inductive phase of anergy (using fixed antigen presenting cells and peptide), neither NF-AT nor one of the NFκB binding factors (NF-κB-B2) was induced (116). In contrast, when the anergized cells were stimulated with peptide and competent antigen presenting cells, normal levels of all the factors associated with IL-2 transcription (NF-AT, AP-1, Oct-1, Oct-2, NF-κB-B1, and NF-κB-B2) were induced, yet the cells secreted very little IL-2 (116). Kang et al. used a chloramphenicol acetyl transferase assay to demonstrate that the AP-1 complex in anergic cells failed to transactivate the phorbol response element of the IL-2 promoter (117). When anergy was reversed with IL-2, normal AP-1 transactivation occured. Gel shift assays showed that significant levels of AP-1 were induced in stimulated anergic cells implying that the defect was due to poor binding of the transcription factor to the promotor (117). The exact mechanism has not been defined.

Anergy in murine T cell clones II: Activation in the absence of IL-2

Anergy can also be induced in Th-1 T cell clones if the autocrine response to IL-2 is blocked. In one such study, T cells were activated with competent antigen presenting cells and peptide, but were prevented from proliferating by the addition of neutralizing anti IL-2 mAb and blocking mAb against the IL-2 receptor. This treatment anergized the cells (118). In a similar experiment, partial anergy was induced if IL-2 was washed out of activated T cell cultures (119).

Anergy in murine T cell clones III: Partial activation with peptide analogues

In a more recent model, Sloan-Lancaster et. al. have shown that peptide analogues can anergize T cell clones (120). T cell clones specific for hemoglobin were

inactivated when they interacted with competent antigen presenting cells and a hemoglobin peptide that had a single amino acid substitution. Similar to the work done with planar membranes (44), it was shown that while the analogue peptide could not induce proliferation or cytokine secretion, it could partially activate the T cell as determined by an increase in cell volume and increased expression of LFA-1 and the IL-2 receptor. It also appeared that this partial activation was necessary for the subsequent anergy as analogue peptides that could not partially activate the cells did not induce anergy. Cyclosporin A could prevent anergy induction in this system, an interesting observation as this drug also prevents anergy induced by fixed cells and peptide (110).

Taken together, all three models of anergy (lack of costimulation, blocking the autocrine IL-2 response, activation with analogue peptides) in murine T cell clones have a common theme, partial activation in the absence of proliferation resulting in subsequent nonresponsiveness. Jenkins (121) has explained the first two models by hypothesizing that partial activation results in the synthesis (or modification) of an inhibitory protein (or proteins) that is diluted out only when the cell proliferates in response to IL-2. If proliferation does not occur, then the inhibitory protein will prevent the full activation of the cell in response to subsequent stimuli. Cleary this explanation can be extended to the third model (activation with peptide analogues) as well but it does not explain why the addition of IL-2 does not prevent the induction of anergy in the first model.

Relevance of anergy in T cell clones to primary T cell anergy

Whereas the work with T cell clones has led to a better understanding of the mechanisms involved in tolerance, it is not clear how relevant work with T cell clones is to tolerance in primary T cells. However, it has been recently shown that immobilized anti CD3 induces anergy in highly purified resting T cells in vitro. After treatment, these cells neither proliferate nor secrete IL-2 in response to

restimulation with the mAb (122). The addition of exogenous IL-2 did not prevent the induction of anergy but interleukin-4 (IL-4) prevented anergy and induced the differentiation of these cells into IL-4 (but not IL-2) secreting Th-2 like cells (122). This corresponded with work showing that Th-1 but not Th-2 T cell clones could be anergized with anti CD3 mAb (122). The investigators did not address the question of whether IL-2 could reverse anergy in primary T cells.

The engagement of the T cell receptor in the absence of costimulation does not always anergize primary T cells. In a recent study, purified naive $CD4^+$ T cells from pigeon cytochrome c specific $\alpha\beta$ TCR transgenic mice were incubated with immobilized MHC class II molecules and the specific antigen (123). The cells did not secrete IL-2 in response to this stimulus but they expressed the IL-2 receptor suggesting that they were partially activated. In contrast to the finding with T cell clones, this partial activation did not lead to subsequent anergy as the cells could later be fully activated if they were given adequate costimulation (123).

Corresponding to the conclusions of in vitro work discussed above, several in vivo studies suggest that immunization with antigen in the absence of costimulation leads to tolerance. Jenkins and Schwartz demonstrated that the intravenous injection of mice with peptide crosslinked to fixed antigen presenting cells would prevent the subsequent activation of T cells when the animals were primed with the same antigen and CFA (103). Furthermore in the mouse experimental allergic encephalomyelitis (EAE) model (where mice immunized with MBP and CFA develop an autoimmune disease similar to multiple sclerosis) Miller et al. (124) showed that injecting mice with myelin basic protein (MBP) coupled to fixed antigen presenting cells prevented disease when the mice were later primed with MBP and CFA. Another group has shown that the injection of mice with soluble MHC proteins complexed to encephalitogenic MBP peptides prevents the induction of EAE when the mice are challenged with the same peptides emulsified in CFA (125).

As mentioned earlier, CD28 has been found to be involved in the costimulation pathway. Its ligand is probably B7, a molecule found on activated B cells(126) and macrophages (127) and expressed on mature splenic dendritic cells (128). CTLA-4, (129) a molecule found on activated T cells (130) is also involved with costimulation and also interacts with the B7 protein (129). CTLA4Ig, a soluble form of CTLA-4, blocks the interaction between CD28/CTLA4 and B7 (129). Lenschow et al. were able to induce specific tolerance to xenogenic islet transplants if the recipient mice are simultaneously treated with CTLA4Ig (131). This molecule, however, can induce only short term acceptance of cardiac allografts in rats (132).

While these studies demonstrate that lack of costimulation can lead to tolerance in vivo, the difficulty of determining whether or not potentially self reactive T cells are present has left unresolved the question of whether anergy is actually the mode of tolerance.

Relevance of anergy in T cell clones to superantigen induced anergy

It appears that the partial activation model cannot readily be used to explain superantigen induced tolerance. To the best of our knowledge, there are no in vitro models of superantigen induced tolerance in murine T cell clones. A.E7, the pigeon cytochrome c (PCC) specific T cell clone used for many of the initial experiments is $V\beta3^+$ and should therefore be reactive to the bacterial superantigen SEA as well as to PCC. It would be interesting to make planar membranes in order to compare the ability of SEA and PCC to induce anergy especially since a rise in intracellular calcium appears to be necessary for anergy induction (109,110) and there have been studies suggesting that superantigens do not induce a calcium flux in T cell clones (98,100).

It is also clear that superantigens induce full and not partial activation of T cells in vivo. They clearly induce proliferation as superantigen administration results in an initial expansion of superantigen reactive T cells (69,72,73). Superantigens also induce cytokine secretion from T cells as serum IL-2 and TNF levels rise dramatically

within an hour of SEB injection (133) and in situ hybridization studies have localized mRNA for these cytokines to the T cell dependent regions of the spleen (134). Thus the mechanism of superantigen mediated tolerance induction in vivo appears to be fundamentally different from the mechanism of anergy induction in T cell clones in vitro.

How do superantigens tolerize mature T cells?

It is still not known why superantigens tolerize mature T cells, although several hypotheses have been proposed. Some light has been shed on this by results obtained in two different systems. When thymectomized Mls 1^- (Mls1b) mice were inoculated with spleen cells from Mls 1^+ (Ms1a) mice, there was an initial phase of clonal expansion of T cells bearing the Mls-1 reactive Vβ6 segment as part of their receptor. These $V\beta6^+$ cells were then largely deleted and the remaining cells did not respond well to Mls1a stimulator cells in vitro (69). Webb et al. proposed the exhaustive differentiation hypothesis based on this work with Mls suggesting that tolerance could be the result of a "powerful" in vivo immune responses.

Work involving TCR transgenic mice specific for the HY (male) antigen has provided another model for peripheral tolerance. CD8 T cells in these mice recognize the HY antigen in the context of D^b and thus proliferate in response to male but not female H-2^b spleen cells. Cells bearing transgenic TCRs are tolerized in male mice but are functional in females (135). When mature naive T cells were taken from female transgenic mice and injected into male nude mice, a phase of clonal expansion followed by unexpected deletion was seen (136). The remaining cells had downregulated levels of TCR and CD8, and responded poorly when challenged with HY^+ spleen cells in vitro. The kinetics of this process were remarkably similar to that seen with SEB (72,73) and Mls (69) primed mice, and the results are consistent with the exhaustive differentiation hypothesis that a powerful in vivo immune response could eventually

lead to tolerance. As the HY antigen is not a superantigen, the tolerance seen in this system suggested that exhaustive differentiation might be a global phenomenon.

Our present results suggest that a prolonged immune response in vivo does not necessarily lead to tolerance. No major changes were seen in the number of KJ1-26$^+$ cells when DO11.10 mice were primed with OVA 323-339. Cells from these mice could respond normally to SEB and to crosslinking of their T cell receptors and were hyperresponsive to OVA 323-339 and ovalbumin. While we have only immunized mice with one dose and looked at one time point, it is still noteworthy that we got a memory type response as treating mice with doses of SEB ranging from as low as 1 ug (70) to as high as 1 mg (71) has always resulted in tolerance and not immunity. Diminished responses are seen even during the clonal expansion phase despite the marked increase in the number of superantigen reactive cells (69, and our own unpublished observations). The development of memory rather than tolerance in response to priming with OVA peptide suggests that exhaustive differentiation alone probably cannot explain SEB induced tolerance. However, in a recent study it was shown that treating preactivated transgenic LN cells with very high doses of specific peptide in vivo (3.2 mg I.V. over a period of 7 days) results in clonal deletion (115). Thus it is possible (though not likely) that very high doses of OVA emulsified in CFA would induce high zone tolerance. It is also possible that very low doses (<1ug) of SEB might induce a memory response.

Another hypothesis proposes that memory cells are inherently non responsive to superantigens and that SEB induced anergy can be largely explained by either the selective deletion of SEB reactive virgin T cells, or the differentiation of SEB reactive naive cells into SEB non responsive memory T cells (137). If superantigen induced tolerance could be fully explained by non responsiveness of memory T cells to superantigens, then one would expect cells from SEB primed DO11.10 mice to respond normally to OVA 323-339 and/or to native ovalbumin; there is no obvious reason why

memory T cells should be anergic to conventional antigens. One might also expect cells from OVA primed DO11.10 mice to be anergic to SEB since memory cells are clearly formed. The fact that cells from SEB primed mice are hyporesponsive to both conventional antigen and superantigens, combined with the fact that cells from OVA primed mice respond normally to SEB, would make the hypothesis of memory cell unresponsiveness unlikely to be the sole explanation of SEB induced anergy.

A third hypothesis stems from a study showing that B cells induce tolerance in virgin T cells in vivo (138). It was proposed that SEB induced tolerance could be the result of the superantigen's ability to bind directly to MHC class II molecules without prior processing. This feature, it was argued, would predispose SEB to B cell presentation since B cells far outnumber professional antigen presenting cells. This B cell presentation would then result in tolerance. Peptides like superantigens can bind directly to surface MHC class II molecules. If superantigen induced tolerance is due to selective B cell presentation, then priming with OVA 323-339 should lead to the same outcome. This was not the case as immunity and not tolerance was seen when mice were primed with the peptide. Thus B cell presentation alone apparently cannot explain the tolerance seen in our system.

It is possible however, that superantigens bind to surface MHC class II proteins on B cells with a much higher affinity than do peptides especially at high doses. Philip Leder's laboratory has recently developed B cell deficient transgenic mice (139). These mice could be used to definitively address the role of B cells in superantigen mediated tolerance. We are currently breeding these mice and plan to test them (in comparison with non transgenic mice) after priming with SEA in order to compare the degree of tolerance in the groups of mice.

It would also be interesting to determine whether dendritic cells, which when pulsed with small quantities of superantigens are potent T cell activators in vitro (140),

would be able to induce tolerance in mature T cells in vivo. This could be done by pulsing dendritic cells with SEB or OVA and injecting them into DO11.10 mice.

What does anergy mean in vivo?

Superantigen induced anergy has been mostly characterized by in vitro proliferation and IL-2 secretion assays. A recent study has suggested that the degree of anergy seen in vitro might not necessarily be indicative of the in vivo response. The investigators tolerized mice with SEB and compared the serum IL-2 levels of control vs tolerant mice in response to SEB restimulation. The tolerized mice produced only 3-5 fold less IL-2 in vivo. In contrast, cells from these mice produced 20-50 times less IL-2 in vitro than control cells. This suggested that the cells were more tolerant in vitro than in vivo (141).

Bandiera et al. have argued that the lack of a proliferative response in Mls-1a tolerized mice does not correlate with in vivo tolerance (142). The investigators found that anergized Vβ6 T cells from Mls-1b mice tolerized with Mls-1a spleen cells were capable of inducing the differentiation of donor Mls1-a B cells into immunoglobulin secreting plasma cells in vitro and in vivo. In addition, anergic T cells appeared to have aquired memory status as they were better able to mediate the rejection of donor Mls-1a B cells in vivo. It was argued that rather than the cells being anergic, they had differentiated into T helper cells whose effector functions included providing help to B cells but not IL-2 secretion or proliferation.

In contrast to this finding, Lussow and MacDonald have recently demonstrated that Vβ8.2 TCR transgenic mice tolerized with SEB produce significantly lower antibody titers than control transgenic mice when immunized and boosted with tetanus toxoid (94). Thus it appears that at least in this model, T cells were functionally anergic in vivo as well as in vitro.

It would be very interesting to study anergy in vivo with the recently developed MBP specific αβ TCR transgenic mouse (143). T cells from these mice express Vβ8.2 as

part of their receptor and should therefore respond to SEB as well as to MBP. T cells from these mice are not tolerant and they develop EAE when their blood brain barriers are transiently permeabilized with pertussis toxin (non transgenic mice develop EAE only when they are immunized with MBP and CFA and given pertussis toxin). It would be interesting to tolerize these mice with SEB and then determine if the anergic cells are still capable of inducing EAE .

Significance of our model

This work, and the previously described study where HY reactive T cells were adoptively transferred into male nude mice (136), are the only two models where a monoclonal population of mature primary T cells have been anergized in vivo. There are other models where TCR transgenic mouse mice were crossed to mice positive for the specific antigen and this generally leads to tolerance in the F1 mice. However the T cell developed in the presence of the "self" antigen and were probably tolerized before they completed development. Our model and the HY antigen model differs from these other models in that mature T cells were tolerized. This is important because Yui et al. have demonstrated that there might be different mechanisms involved in the tolerance of mature and immature T cells (101). It was shown that when T cells from Vβ8.1 TCR transgenic mice were tolerized at an early stage (by breeding the mice on an Mls-1a background), they could neither mobilize calcium nor proliferate in response to anti TCR mAbs. On the other hand, when T cells from the same mice were tolerized after they had fully matured (by treating the mice with Mls-1a spleen cells), they could mobilize calcium but not proliferate in response to the same stimulus. The mechanism involved in tolerizing mature T cells is less well understood but will probably be more relevant to designing specific treatments to combat autoimmune disease and transplant rejection.

In summary, we have demonstrated that when these antigens are emulsified in CFA and are then injected subcutaneously, superantigens and conventional antigens behave very

differently. SEB tolerizes mature T cells whereas OVA peptide induces memory. We feel that this dramatic difference in outcome is most likely due to differences in the signal transduction pathways coupled to the two classes of antigens. The differences between these pathways need to be carefully examined. If superantigens utilize a system that eventually leads to tolerance, then it might be possible to design agents that selectively activate this pathway. Such agents might be useful in treating allergies, autoimmune disease, and transplant rejection.

Chapter 2.1 (Introduction)

The mechanisms involved in T cell activation have been partially worked out. Engagement of the T cell receptor results in initial activation of several tyrosine kinases including $p56^{lck}$ and $p59^{fyn}$. The enzyme phospholipase C is activated by tyrosine phosporylation and this results in the hydrolysis of phosphatidyl inositol bisphosphate into inositol 1,4,5 trisphosphate which produces an increase in intracellular calcium, and diacylglycerol which activates protein kinase C. The two signals activate transcription factors associated with the IL-2 promoter. However, additional signalling through the CD28 coreceptor is required for optimal IL-2 production.

There have been several studies suggesting that superantigens and conventional antigens activate different signal transduction pathways. We felt that these differences might best explain why the interaction of T cells with the two sets of antigens resulted in different outcomes.

Phosphatidyl inositol

O'Rourke et al. (97) first demonstrated differences in the signal transduction pathways triggered by superantigens as opposed to. conventional antigens. The investigators demonstrated that purified Vβ6 T cells that had been primed in vitro with Mls-1a stimulator cells would proliferate but not generate phosphatidyl inositol (PI) hydrolysis in response to a secondary stimulation with Mls-1a cells. The lectin Con A, on the other hand, induced both proliferation and PI hydrolysis. In addition it was shown that alloantigens but not Mls positive cells would trigger PI hydrolysis in a T cell clone and hybridoma that could proliferate and secrete interleukin-2 (IL-2) in response to either antigen.

Oyaizu et al. (100) have recently reported similar findings with a human T cell clone that is specific for a conventional antigen (purified protein derivative, PPD) and a

superantigen (SEB). It was found that PI hydrolsis was generated in response to PPD but not to SEB .

In contrast to these findings, Fraser et al (144) reported that the bacterial superantigen SED, when presented by the Raji B cell lymphoma cell line, would trigger PI hydrolysis in the Jurkat T cell line. The level of hydrolysis seen in response to the superantigen was comparable to that seen in response to anti TCR mAb. This was in agreement with a prior study which demonstrated that the bacterial superantigen toxic shock syndrome toxin 1 (TSST-1) was capable of inducing inositol phospholipid turnover in human peripheral blood mononuclear cells (145).

Calcium flux

Liu et al used mouse T cell clones that were specific for both a conventional antigen (OVA) and a superantigen (SEB) in calcium flux assays and concluded that T cells would mobilize calcium in response to stimulation with conventional antigens but not superantigens (98). This work has been confirmed by another group using human T cell clones specific for PPD and SEB (100). On the other hand, several studies have shown that primary mouse T cells can mobilize calcium in response to Mls antigens (101,146).

CD4

Oyaizu et al. used dual specific T cell clones to demonstrate that that low concentrations of anti-CD4 mAb would inhibit proliferation, IL-2 secretion, and IL-2 receptor expression in response to PPD but not to SEB (100). This supported a prior finding that CD4 deficient hybridomas could respond as well as CD4 transfectants to a panel of bacterial superantigens (147). Recently, however Bhardwaj et al. have found that anti-CD4 mAbs would inhibit the proliferative response of primary human T cells in response to superantigen pulsed dendritic cells (140).

Cytokine secretion

Using murine T cells specific for OVA and SEB, Liu et al. (98) demonstrated that conventional antigens stimulated the secretion of 5-15 fold higher levels of interleukin-3 (IL-3) and IL-2 than superantigens although the two sets of antigens induced similar levels of proliferation. Prior to this the same group showed that conventional antigens but not superantigens would induce transcription of the interferon-γ gene (99). Our own work with primary DO11.10 T cells indicates that IL-2 secretion is induced by SEB as well as by OVA 323-339.

Antigen presentation

Roth et al. (148) recently demonstrated that MHC class II positive astrocytes would present peptide antigen but not superantigens to rat T cell lines. In contrast, bulk thymocytes (which contained dendritic cells) could present both sets of antigens efficiently. It was also shown that macrophages but not astrocytes could present a panel of bacterial toxins to primary mouse T cells. FACS analysis was used to show that the astrocytes were capable of binding the superantigens and it was also shown that they could present superantigen and induce IL-2 secretion in T cell hybridomas. This work suggested that superantigen induced activation required a unique costimulatory molecule(s) that was not present on astrocytes.

CD28/B7(BB1)

The ligand(s) for the CD28 receptor (most likely the B7/BB1 molecule) is the best defined costimulatory molecule for conventional antigens. In reconstitution experiments, Harding et al. showed that immobilized anti-CD3 mAb by itself would not activate highly purified CD4 T cells (107). The addition of either B cell blasts or anti-CD28 mAb to this system would induce proliferation. Importantly, it was shown that the major costimulatory molecule on the B cell blasts was the CD28 ligand as the addition of anti CD28 Fab fragments completely blocked the B cell derived costimulation.

In a somewhat more physiological model, Sagerstrom et al. (123) have recently shown that immobilized MHC class II molecules and peptide would not activate IL-2 secretion T cells from moth cytochrome c specific αβ TCR transgenic mice. The addition of anti CD28 mAb to this system resulted in activation as determined by IL-2 secretion.

There have been conflicting reports on the role of the CD28/B7 pathway in superantigen induced activation. Fraser et. al recently demonstrated that CD28 costimulates SED induced IL-2 secretion in primary human and Jurkat T cells (144). The T cells were transiently transfected with a reporter gene linked to either the wild type IL-2 promoter or an IL-2 promoter with a mutation in the CD28 response element. The mutated promoter responded as well as the wild type promoter when transfected cells were stimulated with either PMA and ionomycin or anti TCR mAbs. However, the mutant promoter responded only 40% as well as the wild type promoter when transfected Jurkat cells were stimulated with SED. In transfected primary T cells, the response of the mutated promoter to a panel of bacterial toxins was only 20% of control suggesting that the IL-2 response of primary T cells to superantigen was even more dependent on CD28 mediated costimulation. In addition, the addition of anti-B7 mAb to the superantigen stimulated transfected cells blocked the activity of the wild type but not the mutated promoter.

Ohnishi et al. showed that anti B7/BB1 mAb inhibited the proliferative response of resting human T cells to the streptococcal superantigen pep M5 presented by autologous antigen presenting cells (149). Furthermore, crosslinking the CD28 molecule would costimulate the response of purified T cells in response to pep M5 in the presence of antigen presenting cell derived culture supernatant. Recently Bhardwaj et al. have shown that blocking mAbs to CD28 can inhibit proliferation of purified T cells by 80-90% in response to dendritic cells pulsed with very small quantities of SEA (140). The system used in this study is probably more physiological as very low doses of superantigen were

used. It is also noteworthy that, under these conditions, other surface molecules such as CD4 and LFA-1 contribute to superantigen induced activation.

In contrast to these findings, Damle et al. (150) reported that costimulation by B7 was not neccessary for the proliferation of human T cells to SEA. Irrespective of whether they were $B7^+$ or $B7^-$, MHC class II bearing cells could induce superantigen mediated proliferation in resting or activated T cells. Moreover, CTLA4Ig, a reagent that binds with high affinity to CD28 ligands and can thus block B7/CD28 mediated costimulation, had no effect on proliferation or IL-2 secretion of T cells in response to superantigens presented by B cell lines. This reagent efficiently blocked the response of T cells to alloantigens presented by the same cells implying that B7/CD28 mediated costimulation was important in conventional antigen but not superantigen induced activation. In this report however, mAbs to CD11a and CD18 (LFA-1) blocked the proliferative response of superantigens presented by various kinds of antigen presenting cells suggesting that the LFA-1/ICAM interaction was essential for superantigen mediated activation. This finding was confirmed by work by Nickoloff et al. (151) where mAbs to LFA-1a, LFA-1b or ICAM-1, but not to B7 were shown to block the response of primary human T cells to MHC class II^+ $B7^+$ keratinocytes.

All these studies were done with human T cells and we were thus interested in determining whether the CD28/B7 pathway played a role in the the response of primary mouse T cells to superantigens. We felt this issue was particularly important as several in vivo studies have suggested that occupation of the TCR in the absence of costimulation leads to tolerance. Thus if the response to superantigens did not require CD28, than this might explain why these antigens tolerized mature T cell in vivo.

Our system offered certain advantages for addressing this issue. DO11.10 cells can respond to SEB as well as to OVA and we were thus able to compare the role of CD28/B7 mediated costimulation in the response of primary T cells to superantigens vs conventional antigens. Two approaches were used in vitro. First we used a B cell line that

expressed very low levels of the B7 antigen (TA3) and its B7 transfected derivative (TA3-mB7) as antigen presenting cells. In addition we examined the effect of anti-CD28 mAb on the proliferative and IL-2 secretion response of DO11.10 T cells to SEB vs OVA.

We found that either anti-CD28 mAbs or high levels of the B7 antigen would costimulate the response of T cells to both SEB and OVA, suggesting that CD28/B7 costimulation plays a role in the signal transduction pathways coupled to either of the two types of antigens.

Chapter 2.2 (Results)

Expression of the B7 antigen

The B cell lymphoma line TA3 expresses very low levels of the B7 as shown in Figure 22. TA3-mB7 was derived in the laboratory of Dr. Casey Weaver by transfecting the TA3 cell line with the murine B7 gene (93). As shown in figure 22, TA3-mB7 cells express significantly higher levels of the B7 antigen than the TA3 parental cell line. Figure 22 also shows that the two cell lines expressed comparable levels of the two MHC class II genes, I-A^d and I-E^d. We compared these cells to the A20 B cell lymphoma line. It was found that A20 cells also expressed very low levels of the B7 antigen and high levels of the I-A^d and I-E^d MHC class II proteins.

Costimulation of the response of purified CD4 cells to SEB or OVA

As shown in Figure 23, the proliferative response of purified DO11.10 CD4 T cells to both SEB and OVA 323-339 presented by the A20 cell line was substantially increased by the addition of anti CD28 mAbs. Notably, anti-CD28 Mab by itself had no effect on DO11.10 cells. Table IX demonstrates that anti-CD28 mAb also increased the amount of IL-2 secreted by the CD4 cells in response to the two antigens presented by the A20 cells.

The addition of anti-CD28 mAb also increased the proliferative and IL-2 secretion response to SEB and OVA 323-339 presented by TA3 cells (Fig. 23 and Table IX). Presentation of SEB and OVA 323-339 by TA3-mB7 cells resulted in significantly increased levels of proliferation (Fig. 23) and IL-2 secretion (Table IX) by DO11.10 CD4 cells as compared to presentation by TA3 cells.

Costimulation of CD8 T cell depleted spleen cells

The proliferative (Fig. 24) and IL-2 secretion (Table X) response of CD8 T cell depleted spleen cells to both SEB and OVA 323-339 was increased by the addition of anti-CD28 mAb. This mAb by itself had no effect on the cells.

Table IX. IL-2 secretion (U/ml) in response to OVA 323-339 (100 nM) or SEB (1 ug/ml) with or without 10 ug/ml of anti-CD28 mAb presented by different antigen presenting cells .

	OVA 323-339 (100nM)	SEB (1 ug/ml)
A20	15	26
A20 + anti-CD28	38	126
TA3	16	99
TA3 + anti CD28	48	277
TA3-mB7	56	240

Table X. IL-2 secretion by CD8 T cell depleted spleen cells in response to OVA 323-339 (100 nM) or SEB (1ug/ml) with or without 10 ug/ml of anti-CD28 mAb.

	OVA 323-339	SEB
Antigen alone	19	29
Antigen + anti-CD28	46	72

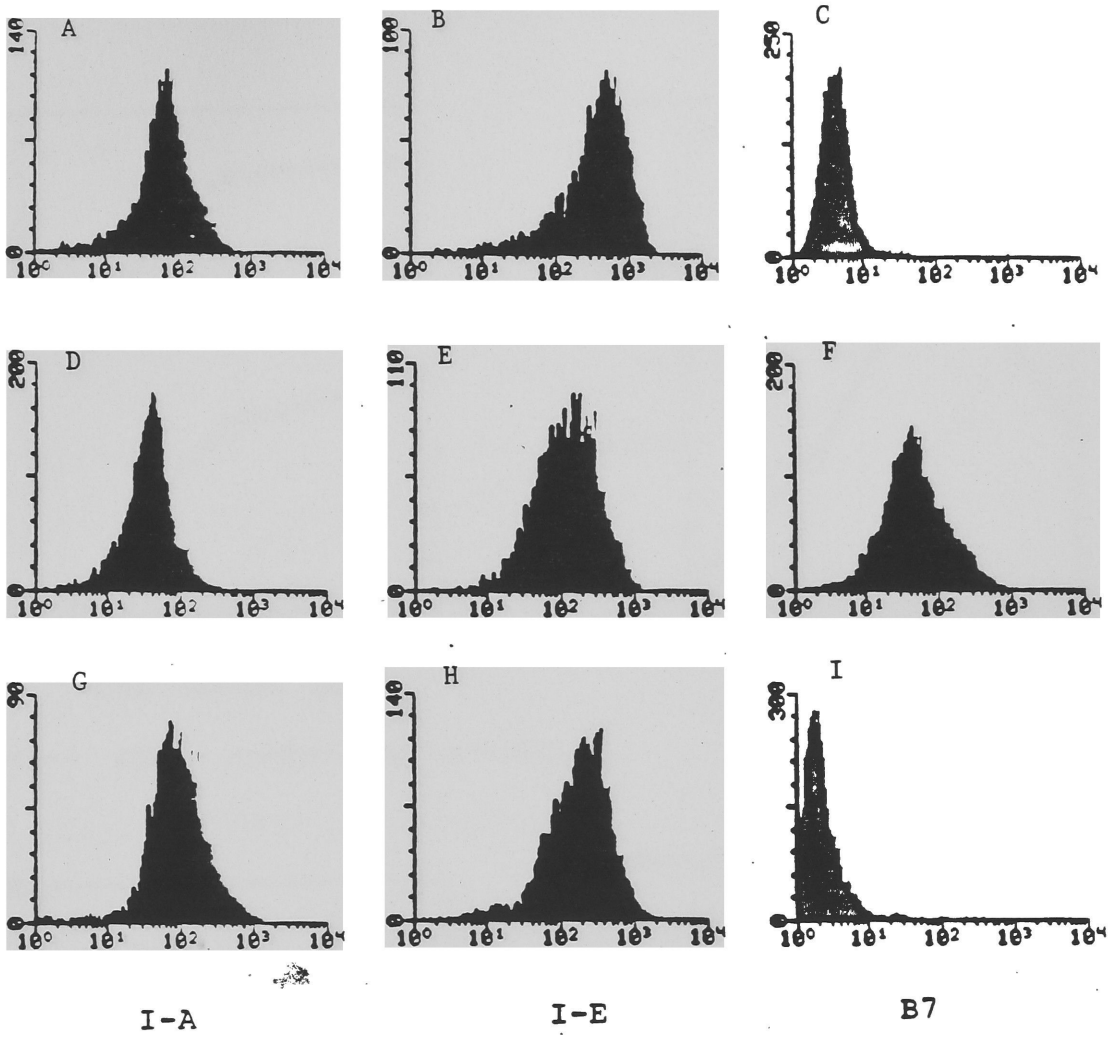


Figure 22. FACS analysis of TA3 cells (A-C), TA3-mB7 cells (D-F) and A20 cells (G-I). Cells were stained with mAb to either IA^d (A,D,G), IE^d (B,E,H) or B7 (C,F,I).

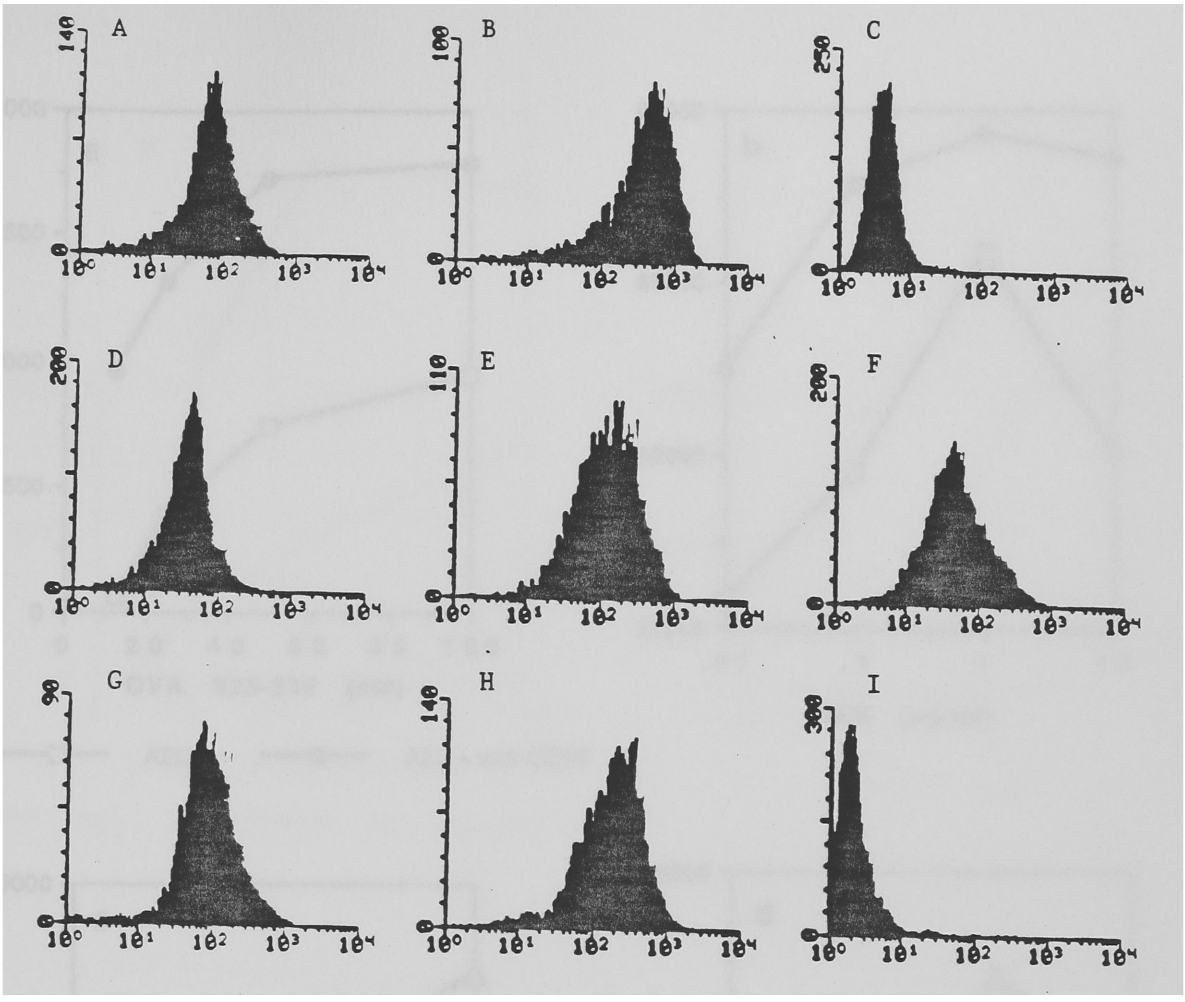


Figure 22. FACS analysis of TA3 cells (A-C), TA3-mB7 cells (D-F) and A20 cells (G-I). Cells were stained with mAb to either IA^d (A,D,G), IE^d (B,E,H) or B7 (C,F,I).

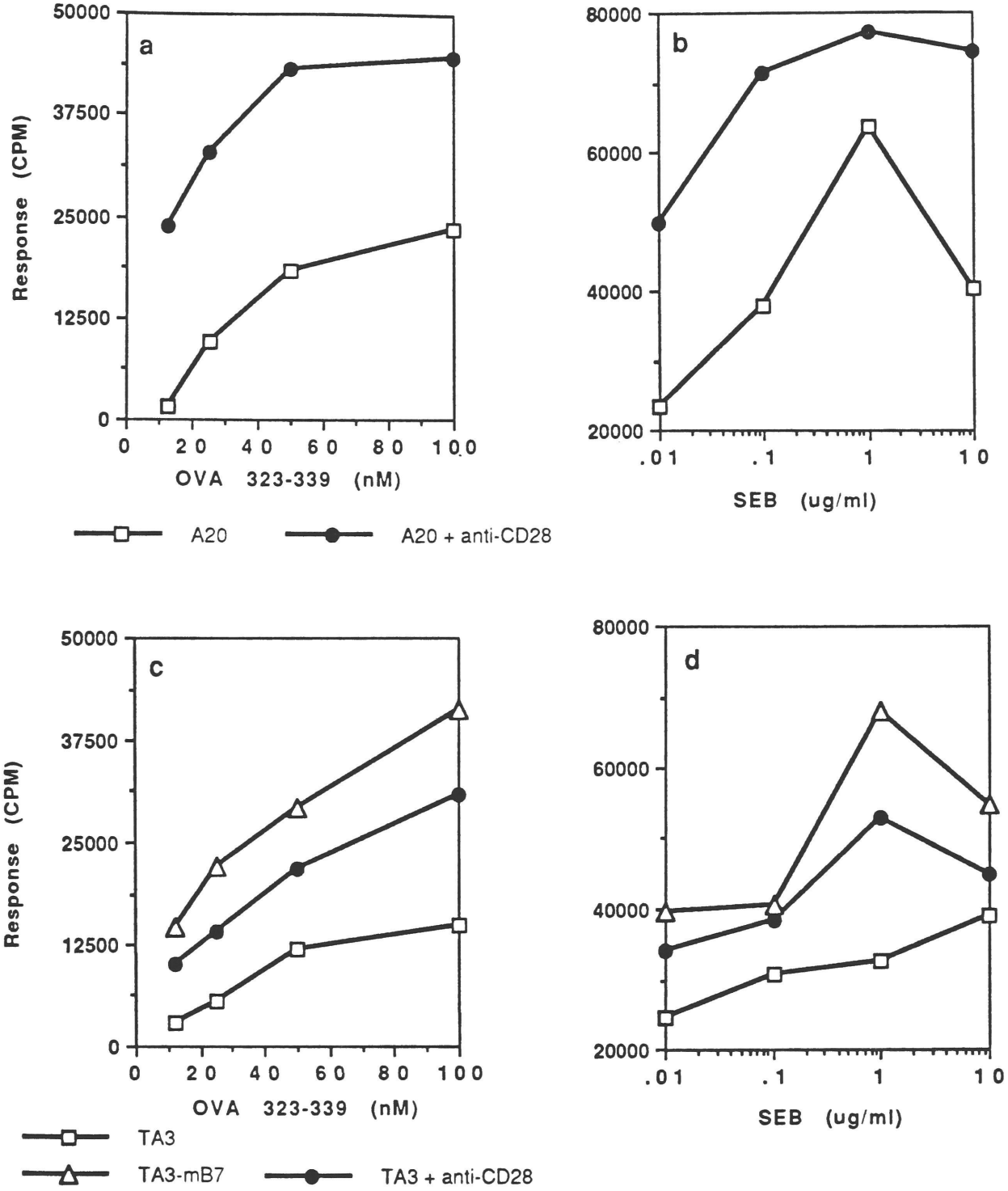


Figure 23. Proliferation of purified CD4 cells from DO11.10 transgenic mice in response to OVA 323-339 (a,c) or SEB (b,d) presented by A20 cells (a,b) TA3 cells (c,d) or TA3-mB7 cells (c,d). Where indicated, anti-CD28 mAb was added at a concentration of 10 ug/ml to each dose of antigen. Background counts (<3200 CPM) were subtracted. Anti-CD28 mAb by itself had no effect on the cells.

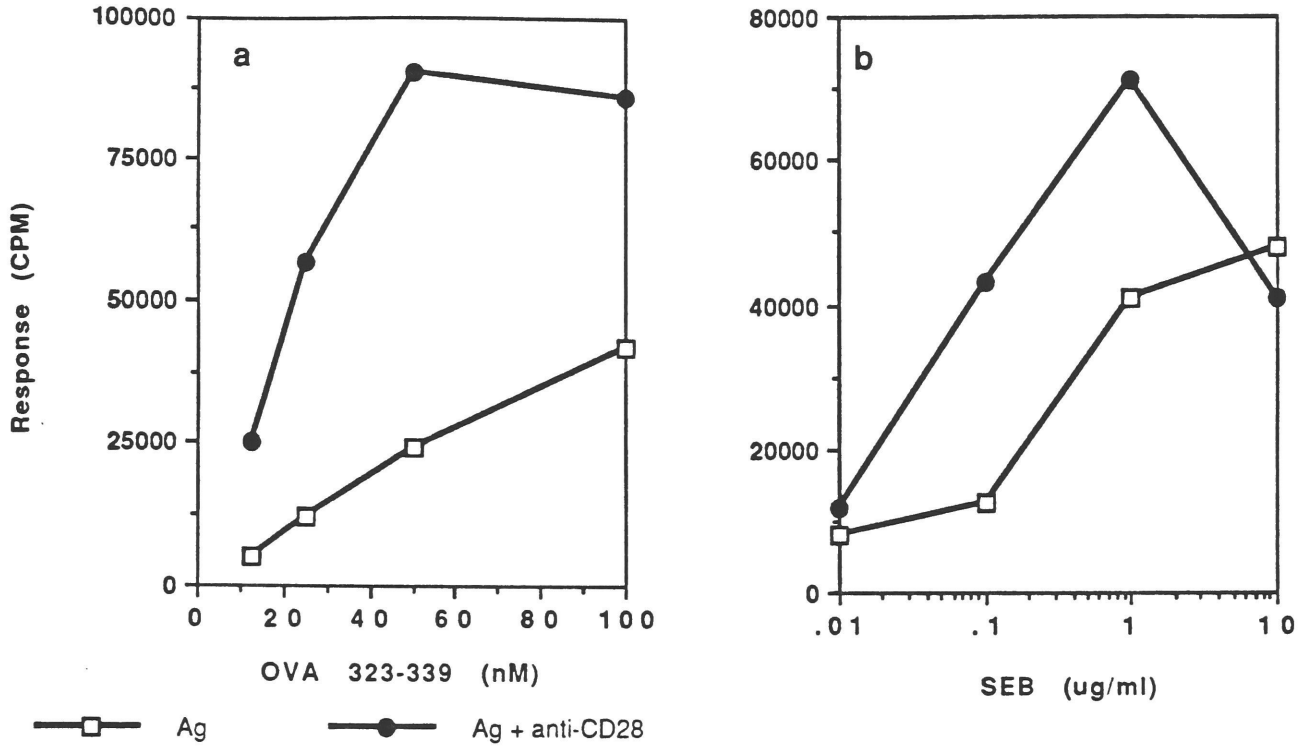


Figure 24. Proliferation of purified CD8 T cell depleted spleen cells from DO10.11 transgenic mice in response to OVA 323-339 (a) or SEB (b). Where indicated, anti-CD28 mAb was added at a concentration of 10 ug/ml to each dose of antigen.

Chapter 2.3 (Discussion)

The CD28 coreceptor was first recognized to have a costimulatory role in human T cells when it was shown that mAbs to this molecule would increase the response of purified T cells to PMA, PHA, anti CD3, or anti CD2 mAbs (152). Studies with a recently developed mAb to mouse CD28 have confirmed that this receptor is involved in costimulation of mouse T cells as well (107, 123).

The counter receptor for CD28 was shown to be B7 by an intercellular adhesion assay using Chinese hamster ovary (CHO) cells transfected with the gene for CD28 (153). This work was later confirmed by showing that CHO cells transfected with the B7 gene were capable of costimulating the response of T cells to anti-CD3 mAb (154).

CTLA-4 has been identified as another receptor for B7 (129). This receptor is found only on activated T cells and at much lower levels than CD28 (130). It however has a much higher affinity for B7 (20 times higher than CD28). CTLA4Ig is a soluble reagent formed from the fusion of the extracellular domain of the human receptor with an immunoglobulin molecule (129). This reagent binds to mouse as well as human B7 molecules and blocks costimulation in both systems.

The previously described reconstitution experiments of Harding et al. (107) and Sagerstrom et al. (123) strongly suggested that CD28 was the major costimulatory molecule involved in mouse T cell activation. This has been confirmed in studies with the recently developed CD28 knockout mice. When T cells from these animals are stimulated with the lectin Con A, the proliferative and IL-2 secretion response is reduced by roughly 80% compared to control T cells (155). The remaining partial response, however, indicates that the signal transmitted through the CD28 coreceptor is not absolutely required for T cell activation (at least in response to Con A).

Recent studies have suggested that there is more than one counter receptor for CD28. Transgenic mice deficient for the B7 gene have been developed (156) and FACS analysis has shown that B cells from these mice still react with CTLA4Ig. In addition, B cells from

these mice could elicit a somewhat reduced allogeneic MLR response which could be partially blocked with CTLA4Ig. This work strongly suggested that there was another CD28/CTLA-4 ligand on B cells.

Hathcock et al. generated a mAb (GL1) that stained activated mouse B cells and inhibited the binding of CTLA4Ig to these cells but did not react with B7 transfected CHO cells (157). Furthermore, this mAb partially inhibited the ability of activated B cells to costimulate the response of purified T cells to anti CD3 mAb. It was also shown that this mAb stained B cells from B7 deficient mice (156) but it has not been determined whether this reagent will block the ability of B7 deficient B cells to elicit an allogeneic response.

As outlined in the introduction, there have been conflicting reports on the ability of the CD28/B7 pathway to costimulate the response of T cells to superantigens. We therefore used primary T cells from DO11.10 mice, that could react to both OVA and SEB, to address this issue. We found that the ability of A20 cells to present both SEB and OVA was enhanced by the addition of anti-CD28 mAbs; proliferation as well as IL-2 secretion in response to both antigens was significantly increased. Anti-CD28 mAbs also had a costimulatory effect on the response of DO11.10 T cells to both OVA and SEB presented by the TA3 cell line. The presence of increased levels of the B7 antigen on TA3-mB7 cells also costimulated the response to the two antigens. The limited response to antigens presented by the low B7 expressing A20 and TA3 cell lines in the absence of anti-CD28 mAb might be due to costimulation with the recently described GL1 ligand (157).

It is important that the addition of anti-CD28 mAb to TA3 cells mimicked the enhanced response of T cells to antigen presented by the TA3-mB7 cell line. This implies that the increased proliferative response to these cells was due to costimulation and not just an increase in the adhesion between responder cells and the transfected cell line.

Anti-CD28 mAbs also costimulate the the response of CD8 T cell depleted spleen cells in response to both antigens. The response seen in the absence of the mAb is probably

solely due to presentation by mature splenic dendritic cells which express high levels of MHC class II molecules and express a CD28/ CTLA-4 counter receptor (128). The CD28 mAb is most likely costimulating the response of T cells to antigen presented by resting B cells which express MHC class II but not B7 (126) nor the GL1 ligand (155).

We felt it was important to address the issue of the role of CD28 mediated costimulation in the response to superantigens since there have been several studies suggesting that engaging the T cell receptor in the absence of costimulation results in tolerance in vivo (103,124,125,131). In particular, It has been shown that treating mice with CTLA4Ig induces specific tolerance to xenogeneic transplants (131). In addition it has been shown that blocking a human MLR reaction with CTLA4Ig (158) in vitro induces tolerance to the alloantigens. It is not known why superantigens tolerize mature T cells in vivo; the ability of these antigens to stimulate T cells without CD28 mediated costimulation might provide an explanation for this outcome.

We have clearly shown that the CD28/B7 pathway costimulates the response to superantigens as well as conventional antigens. We have however not addressed the question of whether superantigens can stimulate T cells in the complete absence of CD28 mediated costimulation. CD28 deficent mice (155) or better still CD28 and CTLA4 deficient mice should be used for this purpose. These mice can be mated to DO11.10 mice and the response of T cells from these DO11.10, CD28 deficient mice to OVA and SEB compared. It would also be interesting to immunize mice with the two types of antigens and determine whether anergy or memory occurs in response to either antigen.

Another way to address this question would be to do reconstitution experiments similar to that of Sagerstrom et al. (123). Primary DO11.10 T cells can be incubated with purified MHC class II molecules complexed to either OVA 323-339 or to SEB. The ability of the cells to respond to the two types of antigens in the presence or absence of mAbs to anti-CD28 or to other putative costimulatory molecules such as LFA-1 can then be compared. In summary we feel that understanding the differences involved in the

signal transduction pathways coupled to the two sets of antigens will eventually lead to an understanding of why in vivo immunization with conventional antigens generally leads to memory whereas immunization with superantigens eventually leads to tolerance.

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