Self-tolerance in the T cell compartment has been attributed to a combination of clonal deletion (negative selection), clonal anergy, and clonal indifference, with regulatory mechanisms such as immune deviation and suppression superimposed on these cell-intrinsic processes. The relative importance of these various mechanisms is thought to depend on the site and avidity of interaction between T cells and antigen. In the thymus, where developing T cells are first subject to selection on the basis of TCR specificity, clonal deletion is thought to be the primary mechanism of tolerance induction (1–4). More recently, deletion has also been shown to operate in the peripheral T cell compartment (5–8).

On the other hand, the precise phenotype and significance of T cell anergy remains more controversial. Originally the term was applied, by analogy with B cells (9), to a state of induced hyporesponsiveness to Th1 cell clones stimulated in vitro under conditions subsequently shown to be associated with suboptimal CD28-mediated costimulation (10–12). The anergic state was characterized by a de-
fect in IL-2 production and proliferation that could be restored by addition of exogenous IL-2 (13). Subsequent use of altered peptide ligand stimulation of T cell clones (14) suggested that a “partial signal” in the presence of normal costimulation could also result in an IL-2-sensitive anergic phenotype.

The term anergy has also been applied to a number of in vivo states of partial or complete unresponsiveness that could not be explained solely on the basis of T cell deletion (for review see reference 15). These states, often induced by exposure to self-antigen, are commonly associated with partial deletion. This has led to the suggestion that deletion, anergy, and indifference represent the response to stimuli of decreasing avidity (16), a model analogous to that postulated for B cells (17, 18). Although anergic B cells show clear evidence of prior antigen recognition in the form of surface IgM downregulation (19), it remains unclear whether T cells rendered anergic in vivo have already made an abortive response to low avidity antigen (the inductive model), or whether low avidity is, of itself, sufficient to encode an anergic phenotype upon primary stimulation of naive T cells in vitro (the selective model). In support of the latter alternative, low avidity stimulation by means of altered peptide ligands has been demonstrated to stimulate naive T cells without inducing sufficient IL-2 to drive proliferation (20). Moreover, in contrast to T cell clones, naive T cells derived from TCR-α/β Tg mice were not rendered anergic in vitro by signaling through the TCR alone (21).

We have examined this question in a double transgenic (Tg1) model in which TCR-α/β Tg mice with specificity for the COOH-terminal peptide of moth cytochrome c (MCC) in association with I-Ek were crossed with antigen Tg mice expressing a fusion protein of hen egg lysozyme (HEL) and MCC (MCC/HEL) (22). In this experimental model, deletion of Tg TCRαβ cells occurs at the double positive stage but is incomplete due to the very low level of antigen expression (23). Previous studies have shown that increasing the expression of the metallothionein-HEL cyt Tg by zinc induction leads to a significant increase in thymic deletion of cytochrome-specific cells (Fazekas de St. Groth, B., and M.M. Davis, manuscript in preparation), suggesting that low avidity allows the exit of self-specific CD4+ T cells into the periphery. However, double Tg mouse show no signs of autoimmunity and have a normal life span, suggesting that the self-reactive T cells are functionally tolerant in vivo. Examination of these cells showed them to be fully responsive to superantigen but poorly reactive to MCC/I-Ek. This phenotype appeared to be due to the expression of two or more TCR α chains paired with the single TCR β chain, causing the cells to be of low avidity for MCC/I-Ek but of high avidity for superantigen. Dual TCR-α-expressing CD4+ T cells derived from naïve TCR Tg mice also displayed an anergic phenotype in response to in vitro stimulation by specific antigen. In other words, low avidity was sufficient to produce an anergic phenotype in vitro, in the absence of prior exposure to antigen. Thus, in this experimental model, selective deletion of high avidity cells in the thymus may fully account for the anergic phenotype.

Materials and Methods

Mice

Tg mouse lines (Table I) and conventional inbred C57BL/6 (B6) and B10.BR mice were bred and housed under specific pathogen-free conditions in the Centenary Institute animal house facilities. All experiments were carried out with approval from the University of Sydney Animal Ethics Committee. TCR Tg mice specific for the COOH-terminal peptide of MCC (22) were created using rearranged Vβ11 and Vβ3 chain genes from the 5C.C7 T cell clone (24) co-integrated and expressed under the control of the endogenous 3′ β chain enhancer (22, 25). Consistent with the phenotype of the previously described cytochrome-c-specific 2B4 TCR Tg mice (26; termed CY in this study), >95% of peripheral T cells expressing the 5C.C7 TCR were selected into the CD4+ compartment. The -D line (22) was established by microinjection of fertile C57BL/6J eggs and maintained by backcrossing to B10.BR mice. Unless otherwise stated, control TCR Tg mice were H-2k/b F1 progeny of -D (B10.BR × C57BL/6) crosses and expressed the 5C.C7 TCR on 60-80% of CD4+ T cells. The remainder of the CD4+ cells expressed an endogenously rearranged α chain paired with the 5C.C7 β chain. 5C.C7 single chain Tg mice (α and β, termed O- and -V, respectively) have been described previously (27). OV mice expressing both TCR chains were derived from crosses of O- and -V single chain Tg mice. Two lines (Y- and Z-) of antigen Tg mice expressing a fusion protein of HEL and the COOH-terminal 24 amino acids of MCC as a soluble neo-self-antigen (termed HEL cyt) under the control of the murine metallothionein promoter were crossed with -D to yield double Tg mice termed YD or ZD (22, 23). In terms of phenotype and function, these two lines were equivalent, showing partial deletion at the CD4+CD8+ stage in the thymus, with 10-40% of residual thymic and peripheral CD4+CD8+ cells expressing the 5C.C7 TCR and the remainder expressing an endogenously rearranged α chain paired with the 5C.C7 β chain.

CD1 Preparation

The thymus and peripheral lymph nodes (pooled superficial cervical, brachial, subcapsular, and inguinal) were harvested aseptically into tissue culture medium (TCM, consisting of RPMI 1640 supplemented with 20 mM Hepes, 10 mM sodium bicarbonate, 50 mg/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 50 μM 2-ME, and 10% FCS [Commonwealth Serum Labs]), and a single cell suspension made by pressing the organs through an 80-gauge stainless steel sieve. Red cell lysis was performed by suspending cells in 0.17 M Tris, 0.16 M ammonium chloride for 3 min at room temperature. Enriching for mature CD4 cells before in vitro culture or sorting was performed by incubating cells with a mixture of mAbs specific for CD8 (3.168; reference 28), HSA (J11d; reference 29), and B220 (RA3.3A1; reference 30), followed by complement-mediated lysis (young rabbit complement, C-six Labs).
Proliferation Assays

Individual cultures from four experimental mice and four controls were routinely assayed in each experiment. Flow cytometric analysis of each cell preparation was performed to allow calculation of the percentage of CD4+ Tg αβ+ cells in each sample. [3H]Tdr incorporation assays with titered numbers of cells were performed in quadruplicate with a starting concentration of 10⁵ cells/well and five twofold dilutions. Irradiated (1,500 rads) syngeneic Tg-negative splenocytes were added at 10⁵ cells/well to serve as a constant source of APCs. The four different stimulation conditions used were: 1 μM MCCC87-103 peptide, 50 U/ml recombinant human IL-2 (rhIL-2), MCC87-103 and rhIL-2 together, and finally TCM alone. For peptide titrations, the number of responder cells was kept constant (10⁵/well), as was the number of irradiated splenocytes (10⁵/well). MCCC87-103 was titrated in 10-fold dilutions as indicated in the figures, and rhIL-2 (50 U/ml) was added where indicated. All cultures were incubated at 37°C, 5% CO₂ for 72 h, and pulsed with 0.5 μCi [3H]Tdr (ICN, Sydney, Australia) in the final 6 h of culture. Before pulsing, 100 μl of culture supernatant per well was collected for cytokine assays. Cultures were harvested and [3H]Tdr incorporation was estimated by a beta scintillation counter.

CFSE Labeling

The intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) was used to label cells (31) before in vitro culture. Thymus and lymph node cells were resuspended at 5.0 × 10⁶/ml in serum-free RPMI 1640 and incubated with 5 μM CFSE for 10 min at 37°C before washing twice with ice cold RPMI 10% FCS. Bulk cultures were set up in 24-well sterile tissue culture plates (Nunc) with 10⁴ cells/well to serve as a constant source of APCs. The four different stimulation conditions used were: 1 μM MCCC87-103 peptide, 50 U/ml recombinant human IL-2 (rhIL-2), MCC87-103 and rhIL-2 together, and finally TCM alone. For peptide titrations, the number of responder cells was kept constant (10⁵/well), as was the number of irradiated splenocytes (10⁵/well). MCCC87-103 was titrated in 10-fold dilutions as indicated in the figures, and rhIL-2 (50 U/ml) was added where indicated. All cultures were incubated at 37°C, 5% CO₂ for 72 h, and pulsed with 0.5 μCi [3H]Tdr (ICN, Sydney, Australia) in the final 6 h of culture. Before pulsing, 100 μl of culture supernatant per well was collected for cytokine assays. Cultures were harvested and [3H]Tdr incorporation was estimated by a beta scintillation counter.

Cytokine Assays

IL-2 Bioassay. IL-2 was measured using the IL-2-dependent CTLL cell line as previously described (32). The standard curve was generated with a known quantity of rhIL-2 (Cetus Corp.). The limit of detection was 0.03 U/ml.

IL-3 Bioassay. IL-3 was measured using the R6X cell line (33). Serial dilutions of culture supernatant were added to 5 × 10⁵ cells in 100 μl TCM and cultured for 42 h before pulsing with [3H]Tdr and harvesting as for T cell cultures. The standard curve was generated using WEHI-3 conditioned medium (34).

IL-4 Capture ELISA. The assay was performed as for the IL-4 ELISA, using mAb 11B11 (38) as the capture Ab and BVD-6-biotin (PharMingen) for detection. The standard curve was generated using recombinant murine IL-4 (a gift from P.D. Hodgkin, Centenary Institute, Sydney, Australia, titered using the HT-2 bioassay in which 1 U/ml was defined as the concentration resulting in 50% of maximal [3H]Tdr incorporation. The limit of detection was 0.03 U/ml.

IFN-γ Capture ELISA. The assay, based on the method of Adorini (35), was performed in 96-well flat-bottomed plates (Immunno Plate Maxisorp; Nunc) using mAb AN.18 (36) for capture, and biotinylated XMG1.2 (37) followed by avidin-peroxidase (Sigma Chemical Co.) for detection. The substrate was 3,3′,5,5′-tetramethyl benzidine (Sigma Chemical Co.) for detection. The limit of detection was 3.3–5.5 ng/ml.

IL-5 Capture ELISA. The assay was performed as for the IFN-γ ELISA, using mAb 11B11 (38) as the capture Ab and BVD-6-biotin (PharMingen) for detection. The standard curve was generated using recombinant murine IL-5 (a gift from P.D. Hodgkin, Centenary Institute, Sydney, Australia, titered using the HT-2 bioassay in which 1 U/ml was defined as the concentration resulting in 50% of maximal [3H]Tdr incorporation. The limit of detection of the ELISA was 7.8 U/ml.

IL-10 and IL-5 Capture ELISA's. These assays are performed using the mAb pairs SXCS1 and biotinylated SXC4 (39) for detection of IL-10, and TRFK5 and biotinylated TRFK4 (40) for detection of IL-5, followed by avidin-peroxidase (Sigma Chemical Co.) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (Sigma Chemical Co.) as substrate. The standard curve was generated with a known quantity of recombinant murine IL-10 or IL-5, as appropriate. (Reagents for this assay were supplied by P.D. Hodgkin). The limits of detection of the assays were 0.098 ng/ml for IL-10 and 31 ng/ml for IL-5.

Antibodies and Flow Cytometry

Tg T cells were identified using a five-color staining protocol, including propidium iodide (PI) to exclude dead cells from analysis. TCR Vα11 was detected with biotinylated R R.81 (41), followed by Texas red-conjugated streptavidin (Molecular Probes). TCR Vγ3 was stained with KJ25 (42) followed by fluorescein-conjugated goat anti-hamster Ig (Caltag), or alternatively with Texas red-conjugated goat anti-hamster Ig (Caltag) for samples containing CFSE-labeled cells. CD8 was detected with αCD8-phycocerythrin (Caltag) and CD4 with αCD4-allophycocyanin (PharMingen) or αCD4-phycocerythrin (PharMingen), as appropriate. In some experiments the protocol was modified to include biotinylated anti-IL-2Rα Ab (PC61; reference 43) detected with allopheyocyanin-conjugated streptavidin (Molecular Probes). All antibodies were diluted in PBS containing 5% FCS and 5 mM sodium azide. Staining was performed in 96-well round-bot-
Published January 18, 1999

tomed plastic plates (ICN Linbro), and incubated on ice in the dark for 30 min. For sterile sorting, the depletion, staining and washing steps were carried out in an aseptic manner. Data were collected on a FACStarPLUS (Becton Dickinson) and analyzed with WinMDI software (Scripps Research Institute, La Jolla, CA). 2 × 10^5 events were routinely collected per sample.

Statistics

An unpaired Student's t test was used to compare V_{\alpha}IL_{\alpha} expression for double and single TCR Tg CD4^+ T cells. The geometric mean fluorescence channel number for 17 samples per group, stained on the same occasion, was log transformed before determining the two tailed P value.

Results

Self-specific CD4^+ T cells in double Tg mice manifest an anergic phenotype. The function of cells bearing the SC.C7 TCR \alpha and \beta chains was examined in vitro by comparing the proliferative capacity of thymocytes and peripheral lymphocytes from double (antigen × D TCR Tg) with that of D TCR Tg mice. Both the number of responding cells per well (Fig. 1 A) and the concentration of specific peptide (Fig. 1 B) were titrated. Thymocytes and lymph node cells from double Tg mice responded to peptide with a 10-fold lower incorporation of ^{3}HITDR than controls. Addition of exogenous IL-2 restored the level of response to that of controls, consistent with the original definition of in vitro T cell anergy by Jenkins et al. (13). The incremental effect of exogenous IL-2 was greatest at low peptide concentrations, when the amount of endogenous IL-2 production would be at its lowest. When thymus and lymph node cell suspensions were depleted of CD8^+ and HSA^+ cells, the results were identical (data not shown), confirming that the proliferative response was attributable to mature CD4^+ cells.

Cytokine production was assessed in supernatants from wells with the highest stimulating peptide concentration in the experiment shown in Fig. 1 B. Supernatants from cultures of double Tg cells contained ~20-fold less IL-2 and 10-fold less IL-3 than the corresponding controls (Fig. 2), again consistent with the classical phenotype of anergy in T cell clones (for review see reference 15). When peptide-dependent proliferation was augmented by addition of exogenous IL-2, secretion of IL-3 and IFN-\gamma by double Tg cells reached a level similar to that of controls (Fig. 2). Production of IL-10, IL-5, and IL-4 was below the limit of detection under these stimulation conditions (data not shown).

Exogenous IL-2 recruits double Tg cells into cell division in response to MCC Peptide. To determine whether the reduction in proliferation of double Tg T cells was due to decreased proliferation per antigen-specific precursor, or to a decrease in the number of precursors recruited into cell division, the intracellular fluorescent label CFSE (31) was used. CFSE-labeled, CD8-depleted thymocytes and lymph node cells were cultured with various concentrations of MCC87–103 peptide with or without rhIL-2 at 50 U/ml. IL-2, IL-3, IL-4, IL-5, and IL-10 were below the limit of detection in the IL-2 and IL-3 assays, for double IFN-\gamma ELISA assays of the CFSE culture supernatants. ND, none detected.

Figure 1. Thymus and lymph node T cells from double Tg mice manifest an anergic phenotype in vitro. (A) Thymocytes and lymphocytes were stimulated under four different stimulation conditions: 1 \mu M MCC87–103 peptide with or without rhIL-2 at 50 U/ml, IL-2 alone, or TCM alone. Four replicate cultures from each of four double and four single TCR Tg H-2^{b /}\beta mice were set up under each condition. Each point represents the mean result for the 16 cultures and error bars represent the SEM. This result is representative of two experiments. (B) Proliferation of thymocytes and lymph node cells in response to titrated concentrations of MCC87–103 peptide. Each point represents the mean result for four double or single TCR, H-2^{b /}\beta mice (16 cultures) and error bars represent the SEM. Where error bars are not visible, they are too small to be apparent. These data are also representative of two experiments, TCM alone; [ ], IL-2; [ ], MCC peptide; [ ], MCC peptide plus IL-2.

Figure 2. Cytokine production by thymus and lymph node cells from double versus TCR Tg mice. IL-2 was measured in supernatants from wells stimulated with 10 \mu M MCC87–103 (from the experiment shown in Fig. 1 B). IL-3 was measured in the same supernatants and in the corresponding wells to which additional rhIL-2 was added. IFN-\gamma was measured in the day 4 supernatants of cultures set up with 10^5 CFSE-labeled cells/ml and 10 \mu M MCC87–103 (see Figs. 3 and 4 for CFSE profiles from these cultures). IL-4, IL-5, and IL-10 were below the limit of detection in the CFSE culture supernatants. Error bars represent the SEM for four individual mice in each group for IL-2 and IL-3 assays, and for duplicate IFN-\gamma ELISA assays of the CFSE culture supernatants. ND, none detected.
MCC peptide, harvested on days 1, 2, 4, or 7 of culture, and stained for cytofluorometric analysis. Data were gated to identify live cells bearing the Tg-encoded specificity (CD4\(^{+}\)V\(_{a11}\)V\(_{b3}\)P\(_{I2}\)). No division was seen on days 1 and 2 in cultures of either double or TCR Tg cells. By day 4, the number of cells recruited into division was lower in cultures containing double Tg cells (Fig. 3), although proliferating cells from both double and TCR Tg mice had undergone an equivalent number of divisions. The number of cells recruited into division (i.e., the inverse of the number in the undivided peak) and the number of subsequent divisions were both functions of peptide concentration in cultures of double and TCR Tg cells. Cell death was not responsible for the decrease in divided cell number in double Tg cultures, as indicated by PI staining (data not shown). Between days 4 and 7, the majority of TCR Tg cells had undergone several further divisions, whereas the fraction of divided cells had not increased substantially in cultures of double Tg cells (data not shown).

When saturating doses of rhIL-2 (50 U/ml) were added to peptide-stimulated cultures of CFSE-labeled thymus and lymph node cells, division of TCR Tg cells was unchanged, whereas recruitment of cells into division in cultures of double Tg cells was increased to a level comparable with that of the TCR Tg controls (Fig. 4). Despite the higher total number of divided cells, the pattern of division was unaffected by addition of IL-2 (see Discussion). In addition, no divisions occurred on the first 2 d of culture even in the presence of saturating IL-2. The day 7 profiles showed that cultures of cells from both TCR and double Tg mice continued to divide in an equivalent manner in the presence of exogenous IL-2 (data not shown).

**Figure 3.** CFSE division profile of CD4\(^{+}\)V\(_{a11}\) thymus and lymph node cells after in vitro stimulation with MCC peptide in the absence of exogenous IL-2. Thymus and lymph node cell suspensions from double and TCR Tg donor mice were depleted of CD8\(^{+}\) cells, labeled with CFSE, and cultured with a titration of MCC\(_{87-103}\) peptide in the presence of a constant number of syngeneic irradiated APCs. Wells with no additional peptide were included as controls. No cell division was seen on days 1 and 2 (data not shown). Histograms of CFSE fluorescence intensity were gated for CD4\(^{+}\)V\(_{a11}\)V\(_{b3}\)P\(_{I2}\) cells and are shown for cultures harvested on day 4. By day 7, the vast majority of TCR Tg cells had entered division, and many had proliferated such that their CFSE signal was too low to be distinguished from the autofluorescence of activated T cells (>8 divisions). In contrast, no further division of double Tg cells was seen between days 4 and 7. The data are representative of three experiments.

**Figure 4.** CFSE division profile of CD4\(^{+}\)V\(_{a11}\) thymus and lymph node cells after in vitro stimulation with MCC peptide and exogenous IL-2. Cultures were set up as for Fig. 3, with addition of rhIL-2 (50 U/ml). Analysis of day 4 CFSE division profiles was performed by gating on CD4\(^{+}\)V\(_{a11}\)V\(_{b3}\)P\(_{I2}\) cells. Once again, no cell division was seen on days 1 and 2 (data not shown). By day 7, the majority of cells in cultures of TCR and double Tg cells had divided at least eight times (the level at which CFSE reaches the level of autofluorescence for activated T cells). The data are representative of three experiments.
Reduced Expression of Tg-encoded TCR α But Not β Chain by CD4+ T Cells in Double Tg Mice. Analysis of Tg-encoded V3,11 and V3,3 expression by mature CD4+ thymus and lymph node cells directly ex vivo showed a small but significant (P < 0.0001) reduction in V3,11 levels on double Tg cells in comparison to control cells (Fig. 5 B, top), whereas the levels of V3,3 (Fig. 5 B, bottom) and CD4 (Fig. 5 A) were identical to that of controls. This suggested that the proportion of mature CD4+ Tg cells expressing dual TCR α chains increases significantly in the presence of cognate self-antigen. The CD44 profile was identical in the polyclonal CD4+ Tg− populations from double and TCR Tg mice, whereas the percentage of CD44hi cells within the CD4+ Tg+ population of double Tg mice was marginally higher than in single Tg controls (Fig. 5 C).

In vitro stimulation revealed an incidental finding underlining the functional consequences of small initial differences in the level of expression of the 5C.C7 TCR by CD4+ cells from double and TCR Tg donors. Division-dependent downregulation of the 5C.C7 TCR caused double Tg CD4+ cells to reach baseline levels prematurely, when compared with TCR Tg controls (Fig. 5 D). Cultures stimulated with peptide and IL-2 were chosen for analysis in order to provide adequate numbers of cells at each division, and cells were gated for IL-2R+ to exclude cells whose initial expression of the 5C.C7 chain was too low to mediate a response to MCC peptide. V3,11 expression by IL-2R+ cells was reduced sequentially at each cell division, suggesting that antigen-mediated TCR internalization occurred at each cell division (44) and/or that de novo synthesis was insufficient to maintain a constant level of expression in daughter cells. The 50% reduction in average V3,11 expression by double Tg cells before stimulation resulted in a rapid decline to undetectable TCR levels after four cell divisions, whereas Tg TCR-α expression on control cells was reduced by only half after the same number of divisions.

Double and TCR Tg Cells Show Equivalent Responses to Staphylococcal Enterotoxin A in the Absence of Exogenous IL-2. Since cells from double Tg mice expressed reduced numbers of 5C.C7 TCRs specific for cytochrome c, it was possible that their hyporesponsive state was simply the result of low avidity for MCC peptide, rather than representational of an induced biochemical change secondary to prior recognition of antigen. To distinguish between these possibilities, the response of double and TCR Tg cells to a V3,3-mediated stimulus, staphylococcal enterotoxin A (SEA), was compared, since double Tg cells expressed the same number of Tg-encoded 5C.C7 V3,3 chains as controls. Thymus and lymph node cell suspensions depleted of CD8+ and HSA+ cells were CFSE-labeled and stimulated in vitro with SEA or MCC peptide. Although the characteristic difference in peptide response was seen (data not shown), the CFSE profiles generated in response to stimulation with SEA were identical for CD4-V3,3-V3,11+ cells from either double or TCR Tg T cells (Fig. 6), and for CD4+ V3,3-V3,11+ cells and CD4+ V3,3-V3,11− cells within each culture (data not shown). In addition, the amount of IL-2 detected in cell culture supernatants was equivalent at each concentration of SEA (data not shown).

To exclude the possibility that V3,3-V3,11− cells may have acted as a source of IL-2 for V3,11+ cells in cultures of double Tg cells, CD4− V3,11+ and V3,11− cells were sorted from thymocyte suspensions derived from double and TCR Tg donors. CFSE labeled, and stimulated in vitro with 100 ng/ml SEA. In this case 100% of CD4+ cells up-regulated IL-2Rα in response to superantigen and the day 3 CFSE division profiles were identical in all cultures (Fig. 7 A). Culture supernatants also showed comparable cytokine profiles, with detection of equivalent amounts of IFN-γ, IL-4 (Fig. 7 B), IL-2, and IL-3 (data not shown). No IL-10 secretion was detected (data not shown). These data show that there is no intrinsic defect in cells from double Tg mice and that they are able to respond normally to TCR-mediated stimuli of sufficient avidity.

Naive T Cells with Low Tg TCR-α Expression Also Manifest an Anergic Phenotype In Vivo. The above data suggested that the anergic phenotype of CD4+ self-specific
cells may have resulted from the reduced number of TCRs available for specific ligand binding. To examine whether naive CD4+ T cells expressing dual α chains also manifested an anergic phenotype in vitro, a number of TCR Tg lineages were compared (see Table I and Fig. 8). To increase the frequency of cells expressing dual α chains, TCR Tg mice expressing either the 5C.C7 (–D) or 2B4 (CY; reference 26) TCRs were bred onto a congenic H-2b background, in which the Tg-encoded specificity was not positively selected. Thus any Tg α1β1 cells reaching the periphery were selected via a second, endogenously rearranged α chain. Comparison of Tg-encoded α with β expression confirmed that the majority of cells in such mice showed significantly less Vα11 staining (Fig. 8, top two rows), but that Vβ3 was expressed at control levels (data not shown).
shown). They also expressed low levels of CD44, indicative of a naive phenotype. Thus, for example, the percentage of CD44hi cells within the CD4+ Tg α+β+ compartment of -D H-2b mice was 1.45 ± 0.04%, compared with 1.16 ± 0.62% for -D H-2k/b mice. CD4+ Tg α+β+ cells from the same donors contained a mean of 3.94 ± 0.38 and 6.45 ± 1.65% CD44hi cells respectively. The CD44 data for the CY line on an H-2b background were similar (Fazekas de St. Groth, B., unpublished data). An anergic phenotype in response to peptide was apparent even though the cells had not been exposed to their cognate antigen in vivo (Fig. 8). In contrast, O V Tg mice on an H-2k background expressed Tg-encoded Vα11 at levels comparable with -D H-2k/b controls and showed no augmentation of peptide-dependent proliferation in response to exogenous IL-2. This example provides further evidence that the anergic phenotype seen here in vitro is a function of the level of Tg-encoded TCR expression, independent of the particular line of Tg mice from which the cells are derived.

**Discussion**

The studies presented here describe a double Tg model of CD4+ T cell self-tolerance induced in the thymus in response to a conventionally processed soluble protein antigen. In contrast to other published studies in which intrathymic recognition of antigen led to complete deletion of developing self-reactive T cells (45), low level expression of the HELcyt neo-self-antigen allowed a proportion of self-specific cells to escape deletion in the thymus and periphery (22, 23). Consistent with the suggestion that low avidity was responsible for escape from deletion, a small but significant reduction in expression of Tg-encoded α but not β chain was detected in residual cytochrome-specific thymic and peripheral CD4+ T cells (Fig. 5). This phenotype, indicative of expression of more than one α chain, has been described previously in several TCR Tg models (46–48) and is known to reduce the avidity for specific antigen, in comparison to cells expressing normal numbers of Tg-encoded αβ pairs (49). Since the dual α, self-specific cells failed to cause autoimmunity in vivo and were hypo-responsive to specific antigen in vitro (22), they provided a unique opportunity to examine nondeletional mechanisms of tolerance in low avidity, self-reactive CD4+ T cells.

Expression of dual α chains by residual self-specific cells was associated with an anergic phenotype after peptide stimulation in vitro, as defined by a decrease in lymphokine production and proliferation per antigen-specific precursor, reversible on addition of exogenous IL-2 (Figs. 1 and 2). This phenotype was also seen in response to stimulation of naïve, dual α-expressing cells from TCR Tg mice (Fig. 8). These data suggest that the anergic phenotype may simply represent the response of low avidity primary cells (the selective model), rather than being the result of repeated low level stimulation in the thymus or periphery (the inductive model).

Several further approaches were used in an attempt to determine whether the anergic phenotype in double Tg animals was best explained by the selective or inductive models. Although the number of self-specific Tg-encoded αβ pairs expressed by anergic cells was reduced, the total number of β chains (and therefore of TCRs) was equal to the number expressed by naïve T cells (Fig. 5 B). Thus the pattern of TCR expression was inconsistent with the changes normally associated with recognition of antigen, namely downregulation of the entire TCR complex via internalization and degradation (44, 50). In addition, cells from double Tg mice did not manifest the small constitutive decline in total TCR and CD4 levels (Fig. 5 A) characteristic of activated and memory T cells (51).

As a further means of distinguishing between the inductive and selective models of anergy induction, cells from double Tg mice were tested for hyporesponsiveness to an antigen different from that to which they had been exposed in vivo. The superantigen SEA was chosen because it is known to be recognized by TCRs using the 5C.C7 Vp3 chain (52), which is expressed at a normal level on 100% of anergic and control cells. The inductive model would predict that downstream TCR signaling would be altered in anergic cells, and hence the response to any TCR-mediated signal should be affected, whereas the selective model would predict a normal response dependent solely on the high avidity of interaction. The responses of cells from double and control TCR Tg mice to SEA were indistinguishable, both being characterized by a normal CFSE division profile (Fig. 6). Sorting of CD4+ Tg β+α+ cells indicated that anergic cells were capable of secreting the same levels of cytokines as control cells in response to SEA (Fig. 7). Thus, the residual self-reactive cells in double Tg mice are not intrinsically unresponsive, nor are they impaired in their ability to produce IL-2 given a TCR stimulus of sufficient avidity.

Our data are thus consistent with the view that a reduction in the number of antigen-specific TCRs accounts for the anergic phenotype in vitro. A twofold reduction in TCR number has previously been shown to exert a profound effect on proliferation in vitro (49, 50) via premature termination of serial triggering and degradation of TCRs (50). This interpretation is also consistent with the characteristically tight peak of surface TCR expression by peripheral T cells, in contrast to most other T cell surface molecules apart from CD4, another molecule closely involved in binding to antigen-MHC complexes.

An incidental finding that underlined the consequences of the small initial reductions in specific TCR levels was the progressive decline in TCR levels with cell division in anergic and control cells (Fig. 5 D). Such a mechanism would be predicted to curtail the T cell response to further stimulation by antigen, and its exaggeration in the case of anergic cells provides a graphic illustration of the effect of reduction in the level of Tg-encoded TCR-αβ on the ability to mount a prolonged in vitro response. However, it should be noted that TCR levels do not show a division-related decline after in vivo stimulation of either anergic or control cells (Girgis, L., and B. Fazekas de St. Groth, manuscript in preparation). Our data do not answer the question of whether the in vitro reduction is caused by re-
peated antigen recognition and TCR internalization at each cell division or by a failure to synthesize sufficient TCR to maintain the level on daughter cells. The effect on division-related TCR levels of removal of antigen after initial activation in vitro should provide an insight into this question.

CFSE labeling revealed that the decrease in proliferation, as detected by \[^{3}H\]TdR incorporation (Fig. 1) was due to a decrease in the total number of divided cells in cultures from double Tg donors, although the distribution of divided cells within the CFSE profile was the same for cultures of double and TCR Tg cells at each peptide concentration (Fig. 3). Differential cell death was excluded as a cause of the reduction in the number of divided cells by three approaches: a proportionate increase in the number of undivided cells, evident particularly at high peptide concentrations, in the cultures of cells from double Tg mice (Fig. 3), viable cell counts (data not shown), and PI exclusion during flow cytometry (data not shown).

Upon addition of exogenous IL-2, the CFSE profiles of double Tg cells indicated further recruitment of peptide-responsive precursors into division (Fig. 4). Anergic cells thus produce insufficient IL-2 to recruit all the cells that have upregulated IL-2R in response to antigen. By contrast, exogenous IL-2 had no effect on cell division in control cultures, indicating that TCR Tg cells produce sufficient endogenous IL-2 to drive cell division at its maximal peptide-dependent rate. Since addition of IL-2 changes the total number but not the CFSE distribution of divided cells in cultures of double Tg cells (Figs. 3 and 4), the rate-limiting step at suboptimal concentrations of IL-2 is recruitment into cell division, rather than progression to further division. Thus, our data indicate either that further cell division is not IL-2 dependent or, more likely, that the amount of IL-2 required to maintain division is relatively small in comparison to the amount required for the first division. The latter interpretation is also supported by comparison of the CFSE profiles for cultures of double and TCR Tg cells at day 7 (data not shown), in which division of double Tg cells ceased prematurely, whereas addition of IL-2 allowed the cells to continue to divide at the same rate as controls.

Recent evidence has suggested that the critical variable affecting the distribution of T cells within the cell division profile is the time to first division, the rate of subsequent division being constant in the presence of adequate concentrations of growth factor (53). As mentioned above, IL-2 availability controls recruitment into division but not the shape of the division profile. In contrast, the cell division profiles (excluding undivided cells) reveal a role for peptide concentration, such that the average number of divisions decreases as peptide concentration decreases, even in the presence of saturating concentrations of IL-2 (Fig. 4). This indicates that peptide concentration controls the response to IL-2 via the expression of IL-2R. Thus the CFSE profiles reveal dissociation of two downstream effects of TCR ligation: IL-2R expression and IL-2 production. Only IL-2 production is affected in anergic cells, suggesting that a twofold decrease in TCR number is crucial for the production of IL-2 but not IL-2R. Published studies have indicated that IL-2R expression has a Ca\[^{2+}\]-independent component and is principally a function of NF-κB translocation (54), whereas IL-2 production requires a prolonged Ca\[^{2+}\] flux (55-57). Thus, dissociation of TCR-induced expression of IL-2 and IL-2R in anergic cells is consistent with currently accepted concepts of diverse downstream TCR-dependent signaling pathways.

One possible explanation of this finding is based on Lanza\`ecevica\'s model of serial TCR triggering in which TCRs are internalized and degraded after binding peptide-MHC (44, 50). Upon initial contact with antigen, when the number of antigen-specific TCRs would be very much in excess of the number of peptide-MHC complexes, a twofold decrease in antigen-specific TCR number would have little effect on signaling. However, as internalization and degradation of antigen-specific TCRs proceeded, their relative representation as a proportion of total TCRs would decline rapidly in cells expressing more than one TCR α chain. Since TCR signaling appears to require formation of oligomers of TCR - peptide-MHC complexes (58, 59), signaling would be curtailed prematurely as unligated TCRs of the wrong specificity interfered with the formation of stable oligomers. By contrast, in cells expressing only one specificity of TCR-αβ heterodimer, signaling would continue until the absolute concentration of TCRs was too low to allow efficient oligomerization.

Correlation of IL-2R expression with peptide concentration, and of IL-2 production with initial antigen-specific TCR number, thus implies that IL-2R expression is closely related to the magnitude of the early TCR-mediated signals, whereas IL-2 production is crucially dependent upon the duration of signal. This explanation is consistent with the selective model, in that a twofold decrease in TCR accounts for the anergic phenotype without the necessity of invoking biochemical changes. A second possibility, consistent with the inductive model, is that the two arms of TCR signaling are differentially affected by induction of anergy, such that only IL-2 production is targeted. However, this explanation is inconsistent with the data regarding the response of naive dual TCR α chain cells to in vitro stimulation (Fig. 8).

Restoration of IL-3 and IFN-γ production to control levels upon addition of exogenous IL-2 (Fig. 2) is consistent with recent evidence that cytokine production is a function of cell division-dependent differentiation (53). Thus, the effect of exogenous IL-2 is to restore the number of cells at each division to control levels, allowing a normal number of cells to progress to cytokine production with a division-dependent probability.

The vast majority of self-specific tolerant cells in our double Tg mice express CD44 at levels consistent with a naïve phenotype, once again providing support for the selective model (Fig. 5C). Priming by environmental antigens probably accounts for the small percentage of CD44hi cells within the dual α population, since such cells would be expected to express a polyclonal repertoire of TCRs containing endogenous α chains. The level of CD44 expression in this population is similar to that in the poly-
clonal CD4\(^+\)Tg\(\alpha\)\(^-\) populations of both double and TCR Tg mice. Consistent with this interpretation, the CD4\(^+\) Tg\(\alpha\)\(^+\) population from TCR Tg mice, which is not subject to negative selection in the thymus and therefore contains a higher proportion of cells expressing only the Tg-encoded \(\alpha\) chain, contains very few CD44\(^+\) cells (Fig. 5 C).

In contrast, in some models of peripheral T cell tolerance induction tolerant cells express high levels of CD44, indicative of chronic activation (60, 61). In the study of Akkaraju et al. (61), peptide-specific CD4\(^+\) T cells were exposed to a soluble neo-self-antigen in both thymus and periphery, and residual peripheral T cells had upregulated CD44 and downregulated both chains of the TCR, suggestive of a response to peripheral antigen after leaving the thymus. Similarly, unresponsiveness also appeared to have been induced in the periphery in the model of Blackman et al. (62, 63). The mechanism of self-tolerance thus appeared to be different from the double Tg model described here. In addition, high levels of IL-10 production by anergic cells have been noted in a number of studies (64–67), although in others naive cells stimulated in the presence of IL-10 became anergic without producing IL-10 themselves (68).

Since IL-10 is one of the last cytokines to be produced by differentiating CD4\(^+\) T cells (53, 69, 70), IL-10–producing anergic cells may have been primed via continuous exposure to a low avidity stimulus (14, 20, 71), analogous to the conditions under which the cells responsible for infectious tolerance are primed (72). Induction of IL-10 under these circumstances is best explained by the inductive rather than selective model of anergy. The use of the term anergy in such diverse cases underlines the difficulties with the current terminology, since it currently covers naive cells, terminally differentiated Th2 cells, and those that have undergone induction of "classical" anergy (11) via TCR stimulation.

Peripheral expression of self-specific TCR \(\alpha\) chains at a reduced level per cell has also been noted in class I–restricted TCR Tg models in which high avidity CD8\(^+\) T cells are deleted in the thymus (73, 74). The residual cells manifest a phenotype termed "split tolerance", in which the cells are functionally tolerant in vivo but reactive in vitro. Since in vitro stimulation of CD8\(^+\) T cells in these studies was performed in the presence of exogenous IL-2 to compensate for the lack of CD4\(^+\) T cell help, it is unclear whether such self-tolerant CD8\(^+\) T cells are analogous to anergic CD4\(^+\) T cells. However, previous studies of split tolerance in polyclonal CD8\(^+\) T cell populations suggested that such cells are indeed specifically unresponsive in vitro in the absence of exogenous IL-2 (75). Thus, the mechanism of tolerance in these models of partial thymic deletion is also consistent with the selective model. In several cases, peripheral rather than thymic tolerance in class I–restricted TCR Tg models also appears to result from selective deletion of high avidity cells, leaving a residual population of dual \(\alpha\) T cells (7, 76, 77).

Here, we have shown in a double TCR Tg model that the presence of a neo-self-antigen in the thymus can cause the appearance of peripheral T cells with reduced Tg TCR-\(\alpha\) expression and an anergic phenotype. We hypothesize that the reduction in avidity secondary to low expression of antigen-specific TCRs leads to reduced responsiveness and in vivo tolerance. A second means of producing low avidity TCR stimulation is the use of low affinity altered peptide ligands, which also generate an anergic phenotype by upregulating IL-2R in the absence of optimal IL-2 production (14). Since the anti-self-repertoire is likely to have been purged of high affinity cells in the thymus (78), the peripheral self-reactive population may be in a functional state analogous to that of the anergic double Tg cells in our study, as demonstrated in normal individuals (79). The in vivo regulation of such low avidity cells after challenge with self-antigen will provide a novel and important model of autoimmune regulation.


