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Altered antigen receptor signaling in anergic T cells from self-tolerant T-cell receptor β-chain transgenic mice

(clonal anergy/T-cell activation)

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ABSTRACT **T-cell tolerance to the minor lymphocyte-stimulating antigen Mls-1^a in a T-cell receptor (TcR) V$_\beta$8.1 transgenic line of mice is maintained by both clonal deletion and clonal anergy. Approximately 20–50% of peripheral CD4$^+$ (but not CD8$^+$) T cells isolated from these mice are anergic and fail to proliferate following TcR ligation. We have examined key events in T-cell signaling in peripheral T cells isolated from these mice. In this report, we show that the anergic CD4$^+$ T cells did not mobilize calcium or express receptors for interleukin 2 (IL-2) following TcR ligation. However, the cells retained viability and functional potential because stimulation with phorbol 12-myristate 13-acetate and ionomycin bypassed the block in receptor-mediated signaling and induced IL-2 receptor expression and proliferation of the anergic cells.**

Studies of self-tolerance have been greatly facilitated by the identification of superantigens, a special class of antigens that stimulate from 5% to 30% of T cells, based predominantly on expression of the variable component of the T-cell receptor β chain (TcR V$_\beta$; see refs. 1 and 2). This allows populations of T cells potentially reactive to a given superantigen to be easily monitored by using anti-V$_\beta$ antibodies and provides a powerful approach to studies of T-cell tolerance. One well-documented V$_\beta$/superantigen association is between V$_\beta$8.1$^+$ T cells and the minor lymphocyte-stimulating antigen 1^a (Mls-1^a) (3). Thus, most V$_\beta$8.1-expressing T cells recognize Mls-1^a, essentially regardless of other components of the receptor. Self-tolerance in Mls-1^a-expressing mice is accomplished largely by the clonal deletion of self-reactive thymocytes, resulting in the virtual absence of V$_\beta$8.1$^+$ T cells in the periphery of these mice (3). These and other studies give strong support to a role for clonal deletion in T-cell tolerance (4).

More recently, clonal inactivation, or clonal anergy, has been described as a mechanism of T-cell tolerance (5–15). Whereas encounter of a T cell with foreign antigen bound to molecules encoded by the major histocompatibility complex (MHC) normally results in T-cell activation, encounter of a self-reactive T cell with self antigen/MHC often results in long-lasting nonresponsiveness, or anergy, that leaves the T cells refractory to subsequent stimulation. The molecular basis of the block in T-cell receptor-mediated signaling in these anergic T cells remains undefined. Specifically, it is not known at what point the αβ TcR is uncoupled from the normal biological response. Although receptor-induced interleukin 2 (IL-2) production has been shown to be a major defect in anergic T-cell clones (16), the results of studies on anergic T cells generated *in vivo* have not revealed a consistent pattern to indicate a specific defect (5–7, 9, 10, 12).

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We have generated a V$_\beta$8.1$^+$ β-chain transgenic mouse and genetically introduced the corresponding self-superantigen, Mls-1^a, in order to study mechanisms of tolerance. Whereas virtually all T cells expressed V$_\beta$8.1, <50% of the cells were clonally deleted in the thymus. However, as we have previously shown, 20–50% of the CD4$^+$ cells that escaped deletion became tolerized in the periphery by anergy (11). This was indicated by the ≈2-fold reduction in proliferation of CD4$^+$ (but not CD8$^+$) peripheral T cells from Mls-1^a-expressing β transgenic mice (Mls-1^{a}βTG) compared with non-Mls-1^a-expressing β transgenic mice (Mls-1^{b}βTG) in response to a variety of agents that stimulate T cells via their TcR. We have proposed that this reduction in proliferation is a consequence of the presence of a subpopulation of nonresponsive CD4$^+$ T cells that have been inactivated upon encounter with Mls-1^a in the periphery, rather than due to a generalized reduction in proliferation of all the T cells in the population. The persistence of a population of normally responsive T cells in the Mls-1^{a}βTG mice could be explained if some transgenic T cells had weak (or no) avidity for Mls-1^a, perhaps a consequence of the α chain with which the transgenic β chain is paired.

We have previously shown that the failure of anergic cells to proliferate is not a consequence of reduced levels of expression of TcR or CD4 (11). The nonresponsiveness is also not a result of uncoupling of the TcR from CD3, because the anergic cells also fail to proliferate in response to direct CD3 ligation (11). In the present study, we have examined the coupling of the TcR with key early and intermediate signaling events in these anergic T cells.

MATERIALS AND METHODS

Mice. TcR V$_\beta$8.1 transgenic mice and B10.BR$_{\beta BR}$ (17) mice were bred at the animal facility at the National Jewish Center for Immunology and Respiratory Medicine (Denver). CBA/J, CBA/CaJ, DBA/2, and B10.D2 mice were purchased from The Jackson Laboratory. The transgenic mice (H-2$^{k/k}$, Mls-1^b) were repeatedly backcrossed with CBA/CaJ and then bred with CBA/J or DBA/2 to give Mls-1$^{a/b}$ heterozygous transgenic mice or with CBA/CaJ or B10.D2 to give Mls-1^b transgenic mice. For simplicity, the Mls-1$^{a/b}$-expressing V$_\beta$8.1 transgenic mice are referred to as Mls-1^{a}βTG, and the Mls-1^b-expressing V$_\beta$8.1 transgenic mice are

Abbreviations: Mls-1^a, minor lymphocyte-stimulating antigen 1; TcR, αβ T-cell receptor; IL-2, interleukin 2; IL-2R, IL-2 receptor; PMA, phorbol 12-myristate 13-acetate; V$_\beta$, variable component of the β chain of the TcR; PE, phycoerythrin; $[Ca^{2+}]_i$, intracellular free calcium concentration.

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referred to as Mls-1$^b\beta$TG. T cells were isolated from 2- to 4-month-old mice and assayed for reactivity.

Cell Preparation. Peripheral T cells were obtained from spleen and/or lymph nodes by passage over nylon wool (18). CD4$^+$ and CD8$^+$ T cells were purified by negative panning (19). Thus, T cells were coated with anti-CD8 (53.6; ref. 20) or anti-CD4 (GK1.5; ref. 21) antibodies, respectively, and incubated on plates precoated with goat anti-rat antibodies (100 μg/ml). Nonadherent cells were removed and used for functional assays. The efficiency of purification was determined by flow cytometry. The procedure resulted in populations that were usually >90% pure.

Immunofluorescence Analysis. Peripheral T cells were analyzed for transgene expression (TcR V$_\beta$8.1) within the CD4 and CD8 populations of T cells by two-color fluorescence using biotinylated KJ16 (specific for V$_\beta$8.1 + V$_\beta$8.2; ref. 22) or F23.1 (specific for V$_\beta$8.1 + V$_\beta$8.2 + V$_\beta$8.3; ref. 23) followed by phycoerythrin (PE)–avidin, in conjunction with fluorescein isothiocyanate-coupled CD4 (GK1.5; ref. 21) or CD8 (53.6; ref. 20). Cells were analyzed on an Epics C (Coulter). IL-2 receptor (IL-2R) expression on cultured transgenic T cells was detected by two-color fluorescence using biotinylated antibodies to the p55 chain of the IL-2R (PC61-5.3; ref. 24) followed by PE–avidin and fluorescein isothiocyanate-conjugated anti-V$_\beta$8 antibodies.

Proliferation Assays. Peripheral CD4$^+$ T cells were stimulated *in vitro* with anti-V$_\beta$8 antibodies as follows. Antibody-coated plates were prepared by overnight incubation of KJ16 (100 μg/ml; ref. 22) on plastic at 4°C, followed by extensive washing. CD4$^+$ responding cells (1×10^5 cells and serial 2-fold dilutions) were incubated in the presence of irradiated (1000 R; 1 R = 0.258 mC/kg) splenic cells from nontransgenic Mls-1^b mice. CD4$^+$ cells were also stimulated with phorbol 12-myristate 13-acetate (PMA) (10 nM; Sigma) and ionomycin (0.5 μM; CalBiochem) in the absence of irradiated filler cells. Proliferation was measured after 3 days by a colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (25).

Analysis of Intracellular Free Calcium Concentration ([Ca^{2+}]$_i$). Purified CD4$^+$ or CD8$^+$ peripheral T cells were loaded with indo-1 (2.5 mM; Molecular Probes), as described (26), incubated with anti-TcR antibody at 20 μg/ml (H57-597; ref. 27), and washed three times. The anti-receptor antibody-coated T cells were exposed to goat anti-mouse immunoglobulin (25 μg/ml; Jackson ImmunoResearch), which cross-reacts with hamster immunoglobulin, to crosslink the primary antibody. Ca^{2+} mobilization was monitored by the change in violet/blue absorbance of indo-1 with time on an Ortho cytofluorograph 50H and a model 2150 computer (Ortho Diagnostics) (36, 37). Data are displayed as isometrics, illustrating cell number as a function of time and [Ca^{2+}]$_i$ and as percentage of cells responding. The percentage is that proportion of cells that increased their peak [Ca^{2+}]$_i$ above 200 nM after stimulation.

IL-2R Analysis. CD4$^+$ peripheral T cells (1×10^6 cells per ml) were stimulated *in vitro* with immobilized anti-V$_\beta$8 antibodies (KJ16; 100 μg/ml), in the presence of 5×10^6 irradiated splenic filler cells per ml (1000 R) from B10.BR$_{\beta BR}$ mice. B10.BR$_{\beta BR}$ mice are B10.BR mice onto which the TcR β locus from C57BR has been bred. C57BR has a large deletion in the TcR V$_\beta$ locus, including deletion of V$_\beta$8.1. The preparation and characterization of this mouse strain has been described in detail (17). Thus, using two-color fluorescence (anti-IL-2R/anti-V$_\beta$8), the percentage of IL-2R-expressing cells among the transgenic T cells can be determined, avoiding the contribution of the irradiated filler cells. IL-2R expression was also analyzed after cells were cultured with PMA and ionomycin in the absence of filler cells. Cells were stimulated for 36 h, washed, and incubated in fresh medium for 3–4 h prior to analysis by flow cytometry.

RESULTS AND DISCUSSION

To determine whether anergic CD4$^+$ T cells retain functional potential, we analyzed their response to PMA and the Ca^{2+} ionophore ionomycin. These pharmocological agents can bypass early TcR-mediated events, directly activating protein kinase C and increasing [Ca^{2+}]$_i$, respectively (28). They may mimic additional T-cell activation signals as well (29). The data in Fig. 1 show the characteristic reduction in proliferation of CD4$^+$ T cells from Mls-1$^a\beta$TG mice compared with Mls-1$^b\beta$TG mice after activation with immobilized anti-V$_\beta$8 antibodies (Fig. 1*A*). In contrast, the same two populations of cells responded comparably after stimulation with PMA plus ionomycin (Fig. 1*B*). The ability of PMA and ionomycin to overcome anergy shows that the nonresponding T cells are viable and capable of proliferation but are refractory to TcR-mediated stimulation because the activation cascade is blocked at some point proximal to proliferation.

In an effort to more precisely define the block in signaling in the anergic T cells, we monitored two well-defined intermediate events in T-cell activation—calcium mobilization and the induction of the IL-2/IL-2R system.

A consequence of TcR activation that is initiated within seconds of TcR ligation is an increase in [Ca^{2+}]$_i$ due to release of Ca^{2+} from intracellular stores and the influx of extracellular calcium across the plasma membrane (30). Measurement of Ca^{2+} levels following TcR crosslinking has several advantages over proliferation as an indicator of T-cell activation in that it is an earlier response and the percentage of responding cells within the population can be precisely determined. Therefore, we compared the ability of T cells from Mls-1$^a\beta$TG and Mls-1$^b\beta$TG mice to mobilize calcium upon crosslinking of the TcR. Purified populations of CD4$^+$ and CD8$^+$ T cells were loaded with the Ca^{2+} indicator indo-1 and coated with anti-TcR antibody (H57-597; ref. 27). Calcium levels were monitored after addition of a secondary anti-immunoglobulin antibody to crosslink the TcR, and the percentage of responding cells in the population was determined. The results (Fig. 2 and Table 1) show a 20–50% reduction in the percentage of CD4$^+$ peripheral T cells that mobilize calcium in the Mls-1$^a\beta$TG mice compared with the Mls-1$^b\beta$TG mice. This reduction in the number of CD4$^+$

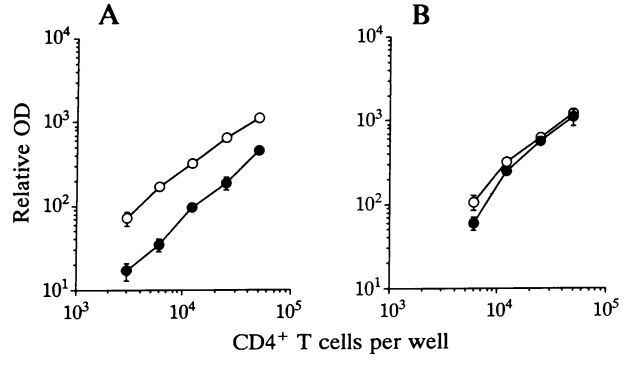

FIG. 1. The nonresponsiveness (anergy) demonstrated by ≈50% of CD4$^+$ peripheral T cells from Mls-1^a-expressing TcRβTG mice after stimulation with anti-TcR antibodies is bypassed by stimulation with PMA and ionomycin. Peripheral CD4$^+$ T cells from Mls-1$^a\beta$TG mice (●) and Mls-1$^b\beta$TG mice (○) were stimulated with immobilized KJ16 antibodies (100 μg/ml; ref. 22) in the presence of 5×10^5 irradiated spleen cells (CBA/Ca; 1000 R) (*A*) and PMA (10 nM) and ionomycin (0.5 μM) in the absence of filler cells (*B*). Proliferation was assessed at the end of 3 days by a colorimetric MTT assay (25) and is plotted as relative optical density at an absorbance of 570 nm. Each point represents the mean of triplicate determinations ± SEM. The cells purified from the Mls-1$^b\beta$TG mice were 91% CD4$^+$ and 94% V$_\beta$8$^+$. The cells purified from the Mls-1$^a\beta$TG mice were 89% CD4$^+$ and 95% V$_\beta$8$^+$.

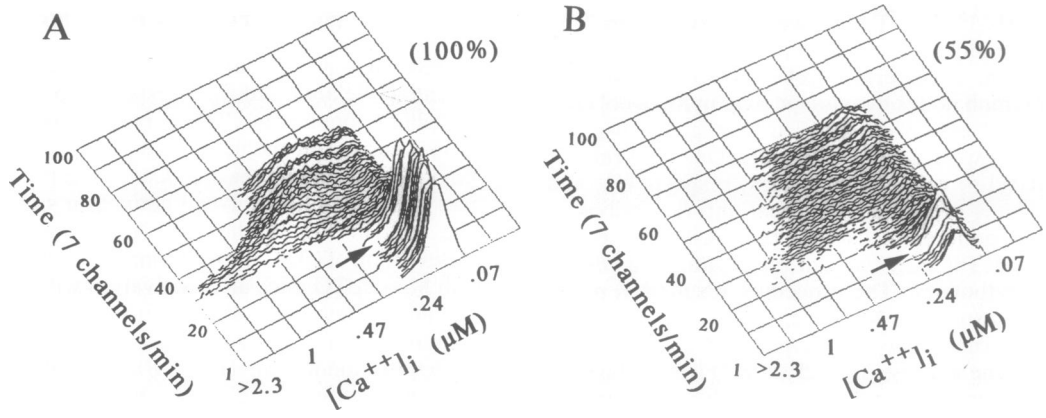

FIG. 2. Approximately 50% of $CD4^+$ peripheral T cells from Mls-$1^a\beta$TG mice fail to increase $[Ca^{2+}]_i$ above the 200 nM baseline upon crosslinking of TcRs. $CD4^+$ peripheral T cells from Mls-$1^b\beta$TG (*A*) and Mls-$1^a\beta$TG (*B*) mice were loaded with indo-1 and incubated with monoclonal antibody to $\alpha\beta$TcRs (H57-597; ref. 27). Flow cytometry measuring indo-1 violet/blue fluorescence ratio as a function of time was initiated to establish a baseline. Subsequently, goat anti-mouse immunoglobulin was added (arrow) to crosslink the antibody-coated receptors. The increase in $[Ca^{2+}]_i$ was measured by monitoring the change in violet/blue fluorescence. The data are presented as an isometric display. The numbers in parentheses refer to the relative percentage of responding cells in the Mls-$1^a\beta$TG population compared with the Mls-$1^b\beta$TG population. The actual percentages of Mls-$1^a\beta$TG and Mls-$1^b\beta$TG responding cells were 42% and 76%, respectively. The cells purified from Mls-$1^a\beta$TG mice were 92% $CD4^+$ and 98% $V_\beta8^+$. The cells purified from Mls-$1^b\beta$TG mice were 92% $CD4^+$ and 94% $V_\beta8^+$.

responding cells could account for the overall reduction in the level of proliferation of the $CD4^+$ T cells from Mls-$1^a\beta$TG mice previously described (ref. 11 and Fig. 1*A*) and thus supports our interpretation that the reduction in proliferation is due to a specific subpopulation of nonresponding cells. The defect in calcium mobilization was confined to $CD4^+$ peripheral T cells because equivalent numbers of $CD8^+$ T cells from the two lines of transgenic mice mobilized calcium (Table 1), again in agreement with earlier proliferation data showing that anergy was confined to $CD4^+$ T cells (11). Also, equivalent numbers of mature $CD4^+$ thymocytes from the two lines of transgenic mice mobilized calcium (data not shown), in support of our previous conclusion that anergy is a peripheral mechanism of tolerance in these mice (11). Finally, anergic cells did not flux calcium in response to crosslinking of CD3 on the cell surface (data not shown), confirming our previous conclusion that anergy is not due to an uncoupling of the TcR from CD3 (11). These data establish that one defect in TcR-mediated signaling in anergic $CD4^+$ peripheral T cells is an inability to increase intracellular calcium.

Table 1. Percentage of peripheral T cells mobilizing intracellular calcium following TcR crosslinking

Exp.	Responding cell population	% responding cells*	Relative % responding cells†
1	Mls-$1^b\beta$TG		
	CD4	76	100
	CD8	72	100
	Mls-$1^a\beta$TG 1		
	CD4	42	55
	CD8	75	104
	Mls-$1^a\beta$TG 2		
	CD4	59	78
	CD8	79	110
2	Mls-$1^b\beta$TG		
	CD4	81	100
	CD8	85	100
	Mls-$1^a\beta$TG		
	CD4	67	82
	CD8	82	96

*Percentage of cells that increase the peak level of intracellular calcium above 200 nM following TcR crosslinking.

†Percentage of responding cells relative to the number of responding cells in the corresponding population from the Mls-$1^b\beta$TG tested in the same experiment.

We next examined another key event in T-cell activation—function of the IL-2/IL-2R pathway. First, we tested the ability of exogenous IL-2 to restore proliferation of anergic T cells, as has been shown in anergic T-cell clones (16), as well as in some (6, 7, 12), but not all (5, 10), *in vivo*-generated anergic T cells. However, in our system, exogenous recombinant IL-2 failed to rescue the response of anergic cells after stimulation of the TcR (data not shown). We cannot yet directly assess the ability of anergic T cells to produce IL-2. We can detect IL-2 in the culture supernatant of Mls-$1^a\beta$TG mice after stimulation (data not shown), but this may be produced solely by the normal (nonanergic) $CD4^+$ cells present in the periphery of Mls-$1^a\beta$TG mice. Therefore, to assess the function of individual cells in this system, we measured expression of IL-2R after stimulation of the T cells, using an antibody specific for the p55 α chain (24). The p55 α chain is a component of high-affinity IL-2R that is not expressed on resting T cells (31). The induction of IL-2R in $CD4^+$ T cells from Mls-$1^a\beta$TG and Mls-$1^b\beta$TG mice was compared after activation with anti-$V_\beta8$ antibodies (immobilized KJ16 plus filler cells; Fig. 3*A*), and activation with PMA plus ionomycin (in the absence of filler cells; Fig. 3*B*). To easily distinguish transgenic T cells from filler cells, we used irradiated filler cells from nontransgenic Mls-1^b-expressing mice ($B10.BR_{\beta BR}$) that do not express $V_\beta8^+$ TcRs due to a deletion in the TcR V_β locus. Cells were analyzed for IL-2R expression early in the response (36 h) to avoid the complication of differential cell proliferation in the two populations. As shown in Fig. 3 *B* and *D*, comparable numbers of $CD4^+$ $V_\beta8.1^+$ T cells from both Mls-$1^a\beta$TG and Mls-$1^b\beta$TG mice expressed the α chain of the IL-2R when stimulated with PMA and ionomycin, stimuli that bypass anergy (88% versus 92%). In contrast, the percentage of $IL\text{-}2R^+$ $CD4^+$ cells from Mls-$1^a\beta$TG mice following stimulation with anti-$V_\beta8$ antibodies was significantly reduced compared with the comparable population of T cells from the Mls-$1^b\beta$TG mice (16.8% versus 44.1%; Fig. 3 *A* and *B*). No $IL\text{-}2R^+$ T cells were detected after culture in medium alone in the presence of filler cells (data not shown). The percentage reduction in $IL\text{-}2R^+$ cells in Mls-$1^a\beta$TG compared with Mls-$1^b\beta$TG mice following anti-V_β stimulation varied between individual mice (40–70%), whereas the percentage $IL\text{-}2R^+$ T cells after stimulation with PMA and ionomycin was always comparable. From this analysis, it is difficult to completely eliminate the possibility that the anergic T cells

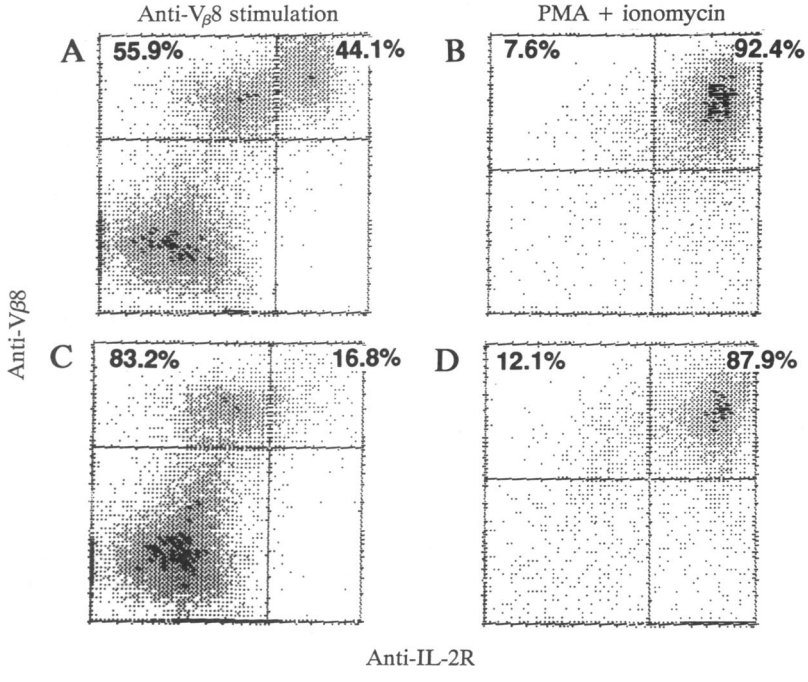

FIG. 3. Induction of IL-2R expression in CD4$^+$ peripheral T cells from Mls-1$^a\beta$TG and Mls-1$^b\beta$TG mice. CD4$^+$ peripheral T cells from Mls-1$^b\beta$TG (*A* and *B*) and Mls-1$^a\beta$TG (*C* and *D*) were incubated with immobilized anti-V$_\beta$8 antibodies (KJ16; 100 μg/ml; *A* and *C*) in the presence of a 5-fold excess of irradiated filler cells or in the presence of PMA and ionomycin in the absence of filler cells (*B* and *D*) for 36 h and tested for IL-2R expression by flow cytometry. Filler cells were obtained from B10.BR$_{\beta BR}$ mice that express the C57BR TcR β locus that has a large deletion, including V$_\beta$8. Thus IL-2R expression of the responding T cells is determined by two-color staining, using a fluoresceinated KJ16 antibody (anti-V$_\beta$8.1 and anti-V$_\beta$8.2), which separates the transgenic T cells from the filler cells, and biotinylated IL-2R (PC61-5.3), followed by PE–avidin.

express low levels of IL-2R. However, there was no detectable shift in fluorescence intensity of the negative cells.

We have shown that a subpopulation of CD4$^+$ T cells from Mls-1$^a\beta$TG mice are anergic in that they fail to proliferate, increase $[Ca^{2+}]_i$, or express IL-2R upon TcR stimulation. It is likely, although not yet proven, that the same population of cells is nonresponsive in all three assays. The addition of IL-2 does not overcome anergy. However, the cells do proliferate and express IL-2R upon stimulation with PMA and ionomycin. We do not know whether PMA and ionomycin are restoring normal TcR-mediated activation pathways or are bypassing the block in proliferation by an alternative pathway.

The failure of the anergic cells to mobilize calcium, express IL-2R, or respond to exogenous IL-2 is in direct contrast to the mechanism of anergy of cloned T cells described in a well characterized *in vitro* system of anergy (16). First, early events in the T-cell signaling cascade including Ca^{2+} mobilization, generation of inositol phosphates, activation of protein kinase C, and the function of at least one tyrosine kinase were found to be normal in the anergic cells (13, 32). Second, the major defect of the anergic T-cell clones was an inability to produce IL-2 (16). This was confirmed by the ability of the cloned T cells to proliferate upon addition of IL-2 to the cultures. However, the cloned T cells may not accurately reflect the state of activation of a normal resting T cell because they express low constitutive levels of IL-2R (33).

Reports on the mechanism of anergy in a variety of *in vivo* models have been conflicting. In some cases, anergic (i.e., nonproliferating) cells have been reported to express levels of IL-2R comparable to normal cells following TcR ligation (5, 6, 9), and the addition of IL-2 has at least partially restored the proliferative ability of anergic T cells (6, 7, 12). Other reports show that whereas IL-2Rs are induced on a percentage of the cells in the population, the anergic cells remain refractory to TcR stimulation upon addition of IL-2 (5, 10).

The conflicting results obtained in the *in vitro* and *in vivo* systems may simply be a reflection of experimental differences in the complex model systems studied. A more interesting alternative is that the variable results reflect multiple pathways of inactivation, perhaps influenced by parameters such as the avidity of the T cell for self antigen, the state of inactivation of the T cell upon encounter with self antigen, or the frequency of encounter with self antigen. A second possibility is that anergy is progressive within a given cell, and the differences reflect degrees of severity of anergy. For example, the requirements for IL-2 production have been shown to be more stringent than those for IL-2R induction (34). Thus, cells that are "less anergic" may retain the ability to express IL-2R but not secrete IL-2, whereas "more anergic" cells may be incapable of expressing IL-2R. Progressive anergy may culminate in the death of the cell and may account for the peripheral clonal deletion that has recently been reported (35).

Understanding the signaling block in anergic T cells will allow experimental manipulation of peripheral tolerance and may have important clinical applications. For example, the ability to induce an antigen-specific anergic state is desirable for controlling graft rejection following transplantation. Also, defining the residual function of anergic T cells and characterizing physiological signals that can overcome the anergic state may contribute to our understanding of some types of autoimmunity.

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