The Effects of Chronic Infection with a Superantigen-producing Virus

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Summary

C3H/HeJ mice transmit a mouse mammary tumor virus from mother to pup in milk. The retrovirus infects mice shortly after birth and, when expressed in recipient mice, produces a Vβ14-specific superantigen. The consequences of such expression on Vβ14-bearing T cells are examined in this paper. Most cells bearing Vβ14 and either CD4 or CD8 are eliminated in the thymus. Some Vβ14-bearing cells escape to the periphery, however. Those bearing CD8 are unaffected by expression of the viral superantigen. The percentage of peripheral $CD4^+$ T cells bearing Vβ14 drops with time after birth. In large part this seems to be due to the fact that many of these cells become anergic because of exposure to the viral superantigen. Unlike normal T cells, these anergic cells cannot undergo peripheral postthymic expansion. Consequently, they drop in percentage even during a time when their total numbers are constant.

In the past, it has been suggested that T cell tolerance could be mediated by one or more of several mechanisms, including clonal deletion, clonal inactivation, and suppression. Evidence for all of these mechanisms has accumulated and it is now clear that under different circumstances different processes occur, all of which result effectively in tolerance. Confrontation of developing T cells with antigen in the thymus usually results in death of the engaged thymocyte (1–4). By contrast, confrontation of mature T cells with antigen in the periphery can result in activation, death, or inactivation of the responding cell (5–9).

For example, several groups have recently shown that in mice challenged with a bacterial or retroviral superantigen, the target T cells first divide and then disappear and/or become inactive (5–9). In these experiments adult mice were usually challenged with fairly large doses of the superantigen in question, either in solution or cell borne. Although these may indeed be "natural" methods of confrontation with superantigens, similar, for example, to infection of mice with a superantigen-producing strain of *Staphylococcus*, we were curious to find out what happens to target T cells in animals challenged by a completely normal route with superantigen. To this end, we have studied the consequences for Vβ14-bearing T cells of infection during nursing with a milk-borne exogenous mammary tumor virus (exoMTV)[1] that carries the gene for a Vβ14-specific superantigen (10). Our results show that target T cells are affected in three ways: by deletion of precursor cells as they mature in the thymus, by deletion in the periphery, and by inactivation. These findings may be comparable with the events that follow infection of animals with other chronic viruses.

Materials and Methods

Mice. Animals were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were thymectomized by standard methods. C3H/HeJ animals transmit the exoMTV in milk, C3H/HeSnJ animals do not.

Cell Culture. Lymph node and spleen cells were separated into $CD4^+$ or $CD8^+$ populations as previously described (7). Cell suspensions were incubated with rat anti-CD4 or anti-CD8 antibodies (GK-1.5 or 53-6, respectively; 11, 12), washed thoroughly, and then panned on plates coated previously with goat anti-rat Ig antibodies. Nonadherent cells were removed after gentle swirling and analyzed for the percentage of CD4- or CD8-bearing cells and for the percentage of cells bearing different Vβs (see below).

These cell suspensions were tested for their ability to respond to different anti-Vβ antibodies as follows. Wells of microculture plates were coated with anti-Vβ antibodies by soaking overnight with a 100-μg/ml solution of the antibody in question. Residual antibody was then removed, and the wells were thoroughly washed with a balanced salts solution (BSS), before 1-h incubation with 10% FCS (FCS/BSS). This was then washed away and replaced with tissue culture medium containing 2×10^5 mitomycin C–treated spleen cells (7) and varying doses of the cell suspensions to be tested for response. Cultures were assayed for T cell proliferation 3–4 d later using the MTT assay (13), an assay that measures mitochondrial activity.

Flow Cytometric Analyses. The percentages of mature T cells bearing CD4, CD8 and/or different Vβs were estimated as previ-

[1] *Abbreviation used in this paper:* exoMTV, exogenous mouse mammary tumor virus.

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ously described (1). Cells were incubated with biotinylated anti-Vβ antibodies, washed, and stained with PE-coupled avidin and fluorosceinated anti-CD4 or fluorosceinated anti-CD8.

To stain thymocytes, these cells were first incubated for 3–4 h at 37°C in tissue culture medium to raise the levels of TCR on immature cells into the clearly detectable range (1). Cells were then incubated with biotinylated anti-Vβ antibodies and trinitrophenylated anti-CD4. After washing, the cells were stained with PE-coupled avidin, allophycocyanin-coupled mAb to trinitrophenol (14, 15), and fluorosceinated anti-CD8. Cells were analyzed on a EPICS 751 with dual lasers (Coulter Electronics, Hialeah, FL).

Results

Consequences of Infection with exoMTV on Peripheral T Cells and Thymocytes Bearing Vβ14. Mice infected neonatally with the exoMTV suffer a slow drop in the percentage of their mature T cells that bear Vβ14. CD4-bearing cells are affected more severely and more rapidly than CD8-bearing cells (Fig. 1) (10). Although most Vβ14-bearing T cells eventually disappear from infected animals, it is worth noting that some survive for very long periods. Presumably, these cells do not bind to the viral superantigen (see Discussion).

To find out whether some or all of this loss might be due to deletion of Vβ14-bearing cells in the thymus, we examined immature and mature thymocytes from mice of different ages. As shown in Table 1. Vβ14-bearing $CD4^+$ or $CD8^+$ cells were at significantly lower percentages in the thymuses of exoMTV-positive C3H/HeJ animals than they were in the closely related exoMTV-negative C3H/HeSnJ mice. This suggests that the milk-borne viral superantigen does reach the thymus and can there cause deletion of Vβ14-bearing T cells.

We have previously shown that all mature thymocytes, and about half of immature thymocytes, are susceptible to clonal deletion if confronted with a ligand that engages their receptors (15, 16). To find out whether the exoMTV superantigen could cause deletion of immature Vβ14-bearing thymocytes, the percentages of cells in this population bearing Vβ14 in infected and control mice were measured (Table 1). Both types of mice contained similar percentages of immature, $V\beta14^+$ cells, indicating that such deletion does not occur. As a control, immature thymocytes from C3H and CBA/CaJ mice were analyzed for the presence of cells bearing Vβ3. C3H but not CBA/CaJ animals contain endogenous MTVs encoding Vβ3-specific superantigens that have previously been shown to lead to deletion of both immature and mature thymocytes (15). As shown in Table 1, Vβ3-bearing cells were at a significantly lower percentage in the immature thymocytes of C3H animals than they were in the same cell population from CBA/Ca mice.

Table 1. *Infection by Milk-borne Virus Causes Deletion of $V\beta14^+$ Cells in the Thymus*

Mouse strain	Vβ	Percent of thymocytes bearing the indicated Vβ among: $CD4^+$, $CD8^+$	$CD4^+$	$CD8^+$
C3H/HeJ	14	4.4	1.3	2.5
CD3/HeSnJ	14	4.8	5.9	8.5
C3H/HeJ	3	1.7	ND	ND
C3H/HeSnJ	3	2.2	ND	ND
CBA/CaJ	3	6.0	ND	ND

Results shown are the average of two animals, except for CBA/CaJ, in which a single animal was analyzed.

Infection with exoMTV Causes Deletion of Mature $CD4^+$ T Cells. Since exoMTV causes the deletion of mature thymocytes bearing Vβ14, it is not surprising that the percentages of peripheral T cells bearing this Vβ drop after birth in infected mice (10). This could occur by gradual dilution of the few $V\beta14^+$ T cells produced before or shortly after birth with $V\beta14^-$ T cells newly produced by the thymus. Alternatively or additionally, it could occur by deletion of peripheral mature Vβ14-bearing cells after expression of exoMTV. To check these possibilities, two types of experiments were done.

In the first, the percentages of $CD4^+$ T cells bearing

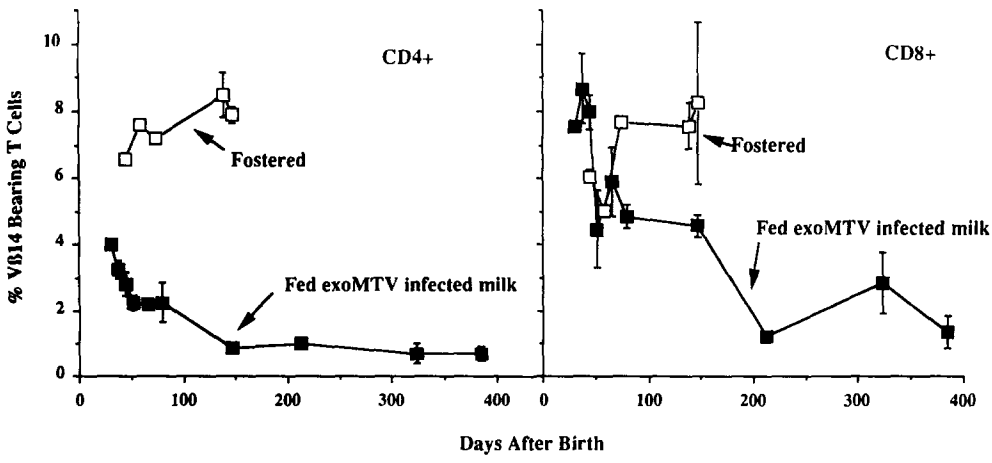

Figure 1. Infection with exoMTV causes the disappearance of Vβ14-bearing T cells. Newborn C3H/HeJ mice were fed by their own mothers, or foster nursed by $exoMTV^-$, (B10.BR × C3H/HeJ)F_1 mice from birth. At intervals thereafter, peripheral blood T cells were isolated from these animals and analyzed for percentages of T cells bearing Vβ14 and CD4 or CD8. (■) C3H/HeJ fed; (□) (B10.BR × C3H/HeJ)F_1 fed. Results shown are the means and SEs for determinations on three mice/group.

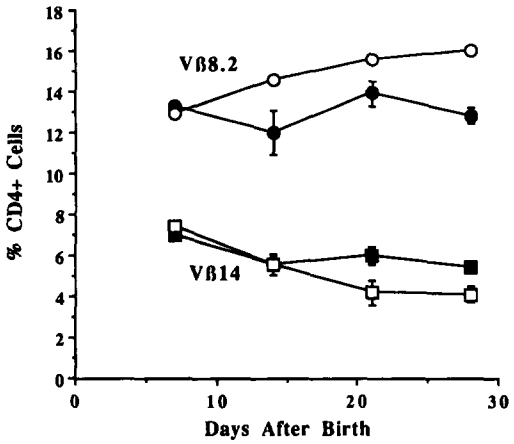

Figure 2. The percentage of Vβ14-bearing cells in the CD4+ population falls with similar kinetics in thymuses and lymph nodes of exoMTV-infected animals. Lymph node T cells and thymocytes were isolated from C3H/HeJ mice of different ages and analyzed for percentages of CD4+, CD8- cells bearing Vβ14 or Vβ8.2. (■) Percent Vβ14+ cells in CD4+ thymocytes; (□) percent Vβ14+ cells in CD4+ lymph node T cells; (●) percent Vβ8.2+ cells in CD4+ thymocytes; (○) percent Vβ8.2+ cells in CD4+ lymph node T cells. Results shown are the means and SEs of determinations on three mice for each point.

Vβ14 in thymus and lymph nodes were measured with time after birth. The results are shown in Fig. 2. The percentages of cells bearing this Vβ fell with the same initial kinetics in the two different locations, suggesting that the disappearance of Vβ14+ cells in the periphery was not completely dependent upon prior intrathymic deletion.

In a second type of experiment, 4–5-wk-old mice were thymectomized and analyzed at various times thereafter for percentages of Vβ14+ T cells. The data in Fig. 3 demonstrate that the percentage of CD4+ cells bearing Vβ14 dropped with time in both thymectomized and sham-thymectomized animals, although not quite as rapidly in the former as the latter. This result suggested that some of the disappearance of Vβ14+, CD4+ cells in normal infected mice is due to dilution with newly formed T cells, but that much of it must be due to loss of superantigen-challenged cells in the periphery.

The results with CD8-bearing T cells were not the same. The percentage of CD8+, Vβ14+ T cells dropped with time in sham-thymectomized mice, but stayed constant in thymectomized animals. This result suggested that the fall in the percentage of these cells was entirely due to dilution of the CD8+ pool with new T cells that specifically lacked Vβ14-bearing T cells, and that the presence of the exoMTV superantigen had no effect on mature cells of this type.

To monitor the effects of thymectomy and/or virus infection on total numbers of Vβ14-bearing T cells, we counted the numbers of spleen and lymph node CD4+ or CD8+ cells in the animals used in this time course. Some of the results for lymph node cells are shown in Fig. 4. Total numbers of CD4+ and CD8+ cells rose dramatically in normal mice between 5 and 7 wk of age. The numbers fell off slowly thereafter. Since the increase in numbers between 5 and 7 wk was less dramatic in thymectomized animals, many of the new T cells must have been produced by the thymus itself, which must be still very active during this period.

The numbers of T cells in thymectomized animals also increased quite remarkably between 5 and 7 wk, however. This probably occurred because of "nonspecific proliferation," continued division of postthymic precursors, a phenomenon that has previously been studied by Stutman and Miller (17, 18).

The percentages of Vβ14-bearing cells in spleen or lymph nodes of the various mouse groups were used in conjunction with the total numbers of T cells to calculate the total numbers of Vβ14-bearing, CD4+ or CD8+ T cells at different times. Sample results for lymph node cells, in Fig. 5, show that regardless of whether or not the animal has a thymus, the total numbers of CD4+ cells bearing Vβ14 hardly increase at all after 4–5 wk of age. Meanwhile, T cells bearing a control Vβ (Vβ2) increase dramatically in numbers in both kinds of mice. In fact, not surprisingly, the changes in numbers of Vβ2-bearing CD4+ cells mirror very closely changes in total numbers of CD4+ cells.

These data suggest that the percentage of CD4+ T cells

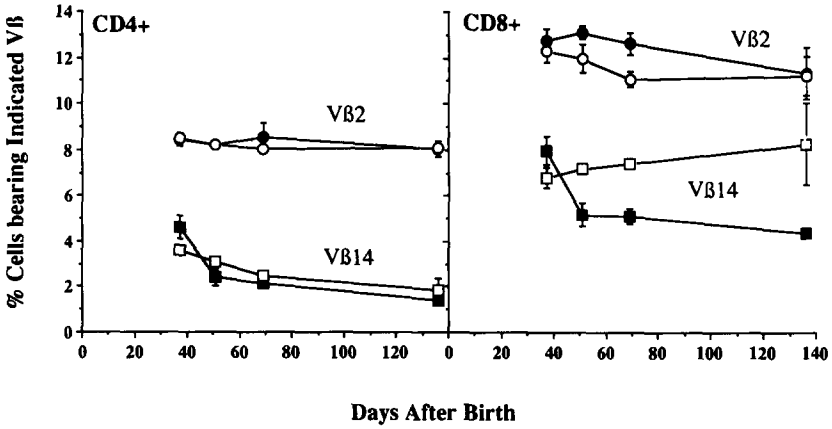

Figure 3. Infection with exoMTV causes a fall in percentage of Vβ14+, CD4+ cells. 32-d-old C3H/HeJ mice were thymectomized or sham thymectomized. At intervals thereafter, three mice from each group were killed and their lymph node cells analyzed for percentages of CD4+ or CD8+ T cells bearing Vβ2 or Vβ14. (■) Percent Vβ14+ cells or (●) percent Vβ2+ cells in normal animals; (□) percent Vβ14+ cells or (○) percent Vβ2+ cells in thymectomized animals. The data shown are the means and SEs obtained for each point.

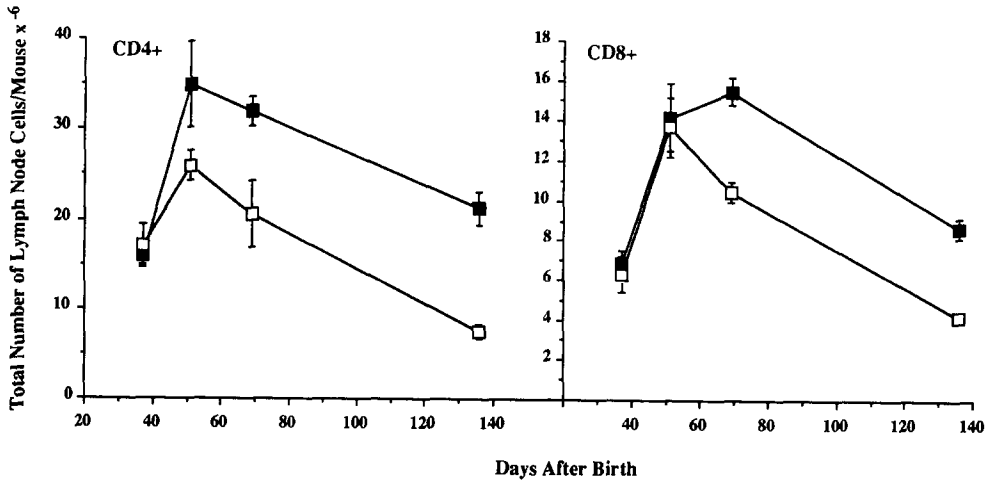

Figure 4. Changes with age in the total numbers of lymph node T cells in normal and thymectomized animals. C3H/HeJ were thymectomized or sham thymectomized at 32 d of age. At intervals thereafter, three mice of each type were killed and the total numbers of $CD4^+$ or $CD8^+$ T cells in lymph nodes counted. Lymph nodes harvested for these analyses were the inguinals, axillarys, brachials, and mesenterics. (■) Total numbers of cells in normal animals; (□) total numbers of cells in thymectomized animals. Results shown are the means and SEs of determinations on three mice for each point.

bearing Vβ14 drops in infected mice not only because the thymus of such animals fails to produce these cells, but also because the V$\beta14^+$, $CD4^+$ cells, unlike other T cells, cannot expand in the periphery.

Infection with exoMTV Causes Inactivation of Mature $CD4^+$ T Cells. Several groups, including our own, have previously shown that mature T cells confronted with superantigens may eventually be inactivated (anergized) by the experience (4–8). V$\beta14^+$, $CD4^+$ cells in exoMTV-infected mice may therefore be unable to undergo postthymic expansion because some or all of these cells are anergic. To find out whether this is so, T cells from exoMTV-infected and control animals were isolated, separated by panning into $CD4^+$ or $CD8^+$ populations, and stimulated with various anti-Vβ antibodies.

As shown in Fig. 6, $CD8^+$ cells bearing Vβ14 from exoMTV mice responded to an anti-Vβ14 antibody just as well as similar cells from exoMTV-negative animals. By contrast, $CD4^+$, V$\beta14^+$ T cells from exoMTV-infected mice responded poorly on a per-cell basis to anti-Vβ14 antibody. This unresponsiveness was particularly pronounced when cells were isolated from 4-wk-old animals. The few $CD4^+$, V$\beta14^+$ T cells left in 8-wk-old $exoMTV^+$ animals responded better, though still not as well as cells from uninfected animals (Fig. 6 and Table 2). This age differential is probably due to the fact, mentioned above, that some $CD4^+$, V$\beta14^+$ T cells cannot react with the exoMTV superantigen at all. These T cells are not eliminated or inactivated by the presence of the superantigen. These cells may undergo postthymic expansion and will certainly not be eliminated because of the superantigen. As T cells that can react with the superantigen disappear, these nonreactive cells will become a larger and larger percentage of the remaining $CD4^+$, V$\beta14^+$ population and, therefore, on a per-cell basis, the population will contain a lower percentage of inactivated cells as the mouse ages.

Discussion

There are many examples of chronic exposure of humans and other animals to particular antigens. Infections such as herpes, Epstein Barr, and the human immunodeficiency viruses come to mind in this context. Although much has been learned about the immune responses to these viruses and their several idiosyncratic means of avoiding or subverting the immune system, little is currently known about the life history of individual virus-responsive T cells while they are constantly

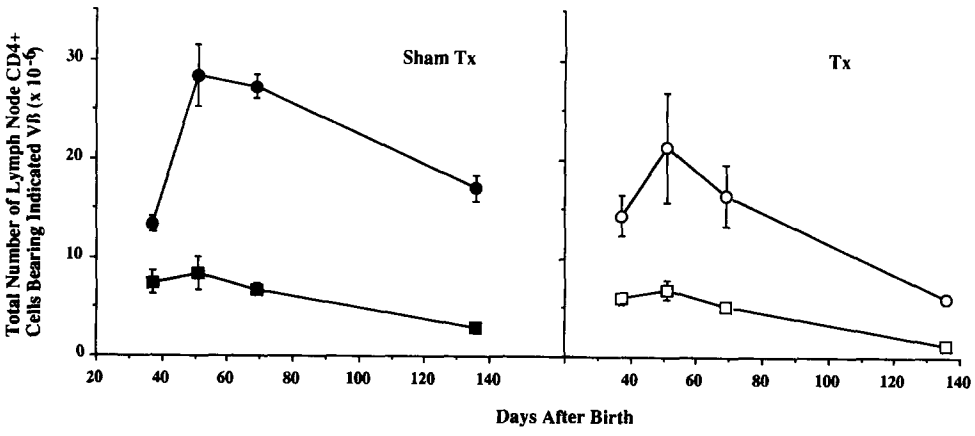

Figure 5. V$\beta14^+$, $CD4^+$ T cells do not undergo postthymic expansion in exoMTV-infected mice. The percentages of $CD4^+$ lymph node T cells in normal and thymectomized mice were measured at different times. These were used, together with the total numbers of $CD4^+$ T cells in the lymph nodes of these animals, to calculate total numbers of T cells bearing both markers. (■, □) Total numbers of $CD4^+$ cells bearing Vβ14; (●, ○) total numbers of $CD4^+$ cells bearing Vβ2. Results shown are the means and SEs of determinations on three mice for each point.

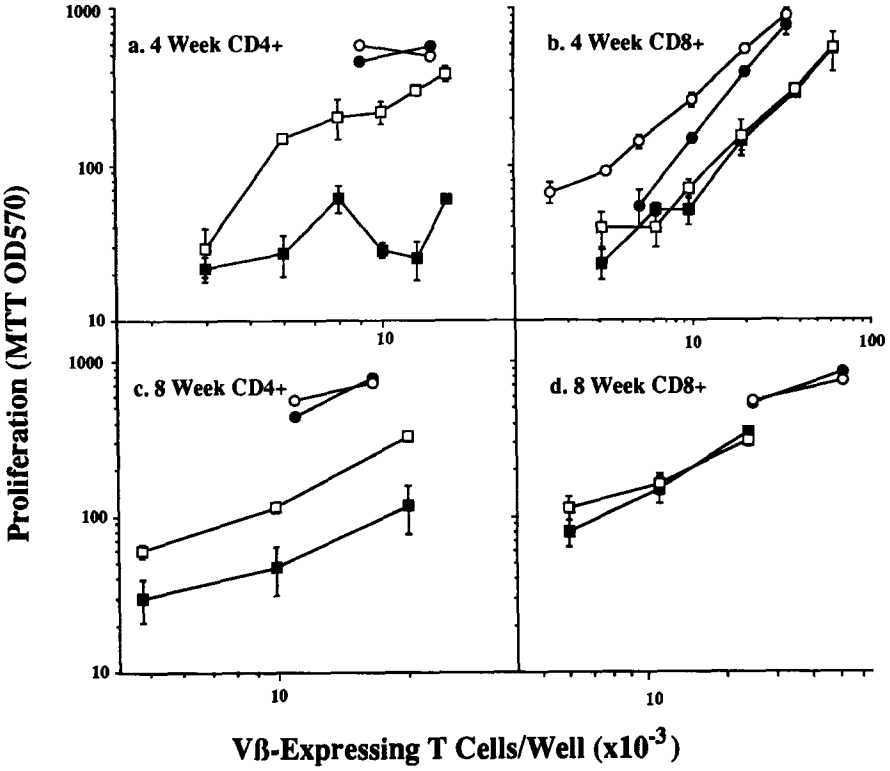

Figure 6. ExoMTV causes inactivation of many Vβ14$^+$, CD4$^+$ cells. T cells were isolated from the lymph nodes of C3H/HeJ or C3H/HeSnJ 4- or 8-wk-old animals. The CD4- or CD8-bearing populations were isolated by panning on anti-CD8 or anti-CD4 plates, respectively, and assayed for responses to anti-Vβ-4, -8, or -14 coated on microtiter wells as described in Materials and Methods. 4 or 5 d later, responses were measured by metabolism of MTT during a 4-h pulse. (*a*) Responses of CD4$^+$ cells from 4-wk-old mice; (*b*) responses of CD8$^+$ cells from 4-wk-old mice; (*c*) responses of CD4$^+$ cells from 8-wk-old mice; (*d*) responses of CD8$^+$ cells from 8-wk-old mice. (■) Responses of C3H/HeJ cells to anti-Vβ14; (□) responses of C3H/HeSnJ cells to anti-Vβ14; (●) responses of C3H/HeJ cells to anti-Vβ4 (*a* and *b*) or anti-Vβ8s (*c* and *d*); (○) responses of C3H/HeSnJ cells to anti-Vβ4 (*a* and *b*) or anti-Vβ8 (*c* and *d*).

being confronted with antigen. In an initial attempt to fill this gap, we have studied the effects of chronic infection with exoMTV on the Vβ14-bearing T cells that can interact with the superantigen produced by this organism. In the "natural" model, we chose mice that were infected neonatally with the virus, transmitted to them in mother's milk.

The data show that response to the superantigen in recipient mice is complex. Direct inspection of thymocytes and experiments involving thymectomy demonstrate that, as virus expression flowers after infection, many precursors bearing the target Vβ are eliminated in the thymus, a phenomenon that has been reported before for many conventional antigens and superantigens (1–3). This occurs presumably because the exoMTV superantigen reaches the thymus and acts, like an endogenous self-antigen, to delete cells that might be self-reactive. Thymus deletion involves T cells bearing CD4 and even cells bearing CD8. In some ways this latter is a surprising result since the thymectomy and stimulation experiments show that mature T cells bearing Vβ14 and CD8 are not affected by the viral superantigen. Since Vβ14-bearing cells are not detectably detectably deleted among the population of thymocytes bearing low levels of TCR, CD4, and CD8, this elimination may happen as immature Vβ14$^+$ cells begin to lose CD4, increase expression of TCR, and turn into mature CD8-bearing T cells. Alternatively, viral superantigen may be expressed at very high levels in the thymus medulla. These high levels may be sufficient to delete Vβ14-bearing, CD8$^+$ cells, cells that are unaffected by the perhaps lower levels of viral superantigen expressed in the periphery.

Some T cells bearing Vβ14 reach the periphery: because they matured before viral superantigen expression began in the thymus, because they bear receptors that cannot interact with the superantigen, or because the superantigen is not expressed at high enough levels in the thymus to affect cells with moderate affinity. The peripheral cells suffer various fates. Thymectomy has no effect on the percentages of cells bearing Vβ14 and CD8, and these cells are not appreciably inacti-

Table 2. *Anergic T Cells Are a Greater Percentage of the Vβ14$^+$, CD4$^+$ Population in Younger Mice*

	Percent response* of C3H/HeJ T cells bearing:		
Age of mice	Vβ4	Vβ8	Vβ14
wk			
4	100.2 ± 0.3‡	ND	13.9 ± 0.5
8	ND	105	40.9

* CD4$^+$ T cells from C3H/HeJ or C3H/HeSnJ 4- or 8-wk-old animals were isolated and titrated for response to anti-Vβ4, anti-Vβ8, or anti-Vβ14 antibodies as shown in Fig. 6. The slopes of the titration lines were used to calculate units of response found in each population. For each experiment the units of response of T cells from C3H/HeJ animals were compared with those of cells from C3H/HeSnJ T cells and expressed as a percentage.

‡ Results shown are the mean and SE of three independent determinations (4-wk-old mice) or the mean of two independent determinations (8-wk-old mice, Vβ14) or a single determination (8-wk-old mice; Vβ8).

vated by the presence of the superantigen. Therefore, as noted above, these cells are unaffected by the virus infection. They probably do not engage the viral superantigen because the avidity of the interaction between $V\beta14^+$ TCRs and MHC plus viral superantigen is not sufficient to trigger the T cell in the absence of additional binding energy provided by the interaction between CD4 and class II. We and others have noticed similar results for peripheral $CD8^+$ cells challenged with other superantigens (5, 10).

T cells bearing CD4 and $V\beta14$ suffer one or more of three fates: they may be unaffected, inactivated, or deleted. The few $V\beta14$-bearng, $CD4^+$ cells that are unaffected by exoMTV presumably bear receptors that cannot interact with the viral superantigen. This may be because the other variable components of the TCR they bear interfere with recognition of viral superantigen plus MHC. Alternatively, binding of $V\beta14$ to this superantigen may be weak, and additional contributions to binding may be required of the other variable components of the TCR. On some cells the other TCR variable element ($V\alpha$, $J\alpha$, etc.) may not have enough affinity for MHC plus superantigen to provide this additional contribution.

On the whole, however, the percentages of peripheral T cells bearing $V\beta14$ and CD4 drop with time. In large part this seems to be due to the fact that most peripheral T cells divide after they have emerged from the thymus. This phenomenon of postthymic expansion has been well documented before (17, 18), but is not completely understood or often taken into account in studies of the type described here. The expansion does not seem to be necessarily antigen driven, and may instead be a response of newly produced T cells to some kind of homeostatic signals, signals that control the total numbers of T cells in any given animal. Most of the $V\beta14^+$, $CD4^+$ peripheral T cells are anergic and do not appear to participate in postthymic expansion. Hence, their percentage in the total T cell pool drops, even though their total numbers remain relatively constant.

Analysis of C3H/HeJ $V\beta14^+$ T cells from mice of different ages indicated that a greater percentage of these T cells are anergic when the mouse is young (4 wk old) than when it is older (8 wk old). There are several explanations for this result. Anergic T cells may have a shorter half-life than normal cells, and therefore tend to disappear with age, constituting a smaller percentage of the surviving $V\beta14^+$, $CD4^+$ pool. Alternatively, the $V\beta14^+$, $CD4^+$ cells that are unaffected by the exoMTV superantigen will undergo postthymic expansion and therefore outgrow their anergic fellows.

Overall, there is relatively little evidence in this system for deletion of mature T cells by contact with the viral superantigen. For example, the data in Fig. 5 show that the numbers of $CD4^+$ T cells bearing $V\beta14$ remain quite constant in mice from 32 to 70 d of age, even if the animals are thymectomized. This is true even though a large proportion of these cells are anergic. It is difficult to draw a firm conclusion from such a result because postthymic expansion of the few $V\beta14^+$, $CD4^+$ T cells that are not anergic might compensate for losses due to rapid death of inactivated cells. One can obtain some estimate of how much such compensation occurs from the fact that, even in 8-wk-old mice, about 60% of the cells in this population are still anergic (Table 2). Therefore, chronic exposure to the exoMTV superantigen does not appear to cause dramatic deletion of target T cells and, on the whole, the half life of anergized T cells does not seem to be markedly shorter than that of normal T cells.

In other systems, inactivation or deletion of mature superantigen-reactive T cells by acute confrontation with superantigen is preceded by activation of these same cells. A similar phenomenon has been reported when T cell lines or clones are exposed to conventional antigens in vitro. It has been suggested that activation is a necessary precursor to inactivation and deletion of mature T cells, and that the latter events represent some sort of "clonal exhaustion." There is no sign of an acute activation of $V\beta14$-bearing T cells, monitored by the presence of IL-2 receptors on $V\beta14^+$, $CD4^+$ T cells, in mice infected neonatally with exoMTV (data not shown). We cannot, however, conclude that in such animals mature T cells are inactivated without being previously activated by the viral superantigen. Presumably, superantigen expression occurs slowly and asynchronously in the virus-infected baby mice. Therefore, only a small percentage of target T cells may be activated at any one time, and this percentage may be too low to be detectable by cytofluorographic analyses.

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