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T cells with two functional antigen-specific receptors

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ABSTRACT Although the clonal selection theory states that lymphocytes should bear only a single specificity of receptor, there is much evidence that some T cells, at least, bear two receptors. Here, we have used mice transgenic for genes encoding an autoreactive T-cell receptor (TCR) to examine the specificity of T cells bearing two functional TCRs. We find that T cells developing in mice that do not express the major histocompatibility complex (MHC) molecule recognized as self by the transgene-encoded TCR express both this TCR and a second TCR that is specific for the MHC molecules of the strain in which it arose. Thus, these T cells have two TCRs, each specific for a distinct antigen bound to a distinct MHC molecule. In contrast, when raised in mice bearing the MHC for which the receptor is specific, T cells develop that express the transgene-encoded TCR almost exclusively. Such mice are highly susceptible to autoimmune disease. Our data suggest that on most T cells bearing two TCRs, only one is specific for peptides bound to self-MHC molecules and, thus, that expression of two TCRs does not usually confer reactivity to two unrelated antigens.

Lymphocytes bear clonally distributed receptors for antigen, and adaptive immune responses are mounted when the receptors on individual lymphocytes recognize antigen borne by professional antigen-presenting cells (APCs), triggering the lymphocytes to proliferate and differentiate into effector cells. Recently, it has been shown that some T cells bear two antigen receptors with a common β chain and two distinct α chains (1, 2), and it has been suggested that this dual reactivity violates a central tenet of clonal selection theory, the monospecificity of lymphocytes. It has also been proposed that T cells with two T-cell receptors (TCRs) may contribute (*i*) to autoimmunity, where one receptor responds to foreign antigen and the other to self, or (*ii*) to alloreactivity, where one receptor responds to peptides bound by self-major histocompatibility complex molecules (MHC) and the other to nonself-MHC molecules (2). To further examine the development of T cells with two TCRs, we have analyzed $CD4^+$ T cells in mice transgenic for the rearranged genes encoding the α and β chains of an autoreactive TCR specific for myelin basic protein (MBP) and the MHC class II molecule I-A^u (3). This system has allowed us to examine the expression of TCRs of two different types on a single cell and to ask directly whether T cells having two different TCRs are required for the development of autoimmunity. While we do find T cells bearing two different TCRs in this system, two findings argue against this being a violation of monospecificity and, thus, a potential contributor to autoimmunity. First, when the TCR transgene is expressed in mice that also express the self-MHC molecule for which the TCR is specific, strong positive selection occurs, and there is little evidence for expression of TCRs other than that encoded by the transgene on peripheral T cells. Moreover, these mice are highly susceptible to autoimmunity mediated by this TCR. Thus, autoimmune disease does not require T cells expressing two TCRs. Second, T cells expressing two TCRs are prominent only in mice that do not express the MHC molecule for which the autoreactive receptor is specific, and in these mice the transgene-encoded TCR, although expressed, is not able to cause disease. Thus, T cells appear to express two TCRs mainly when one of them cannot recognize peptides bound to self-MHC, and such TCR also would not be able to mediate responses either to self or to nonself peptides bound to self-MHC.

MATERIALS AND METHODS

Transgenic Mice Expressing MBP-Specific TCR. Mice transgenic for the TCR of an I-A^u-restricted, $CD4^+$, $V\beta 8.2^+$ T-cell clone specific for the acetylated N-terminal 16-amino acid fragment of MBP [Ac-MPB-(1–16)]—clone 19 (described in ref. 3)—were generated by cloning the rearranged α and β TCR chain genes of clone 19 from genomic DNA along with regulatory elements for T-cell expression into cosmids and introducing them into the germ line of C57BL/6 mice (4); $V\beta 8.2$ refers to a TCR β-chain variable (V) region. The transgene-positive mice were crossed to PL/J and B10.PL mice (*H-2^u*) as well as C57BL/6 (*H-2^b*) that were purchased from The Jackson Laboratory. The mice were typed by screening peripheral blood for the presence of the MBP-specific TCR by staining with the anti-clonotypic antibody 19G (3) and fluorescein-activated cell sorting (FACS) analysis. For MHC typing, monoclonal antibodies (mAbs) Y-3JP (against I-A^b, I-A^u), Y-25 (against K^b), and Y-17 (against $E\beta^bE\alpha$) were used to distinguish between mice that are homozygous for *H-2^u* or *H-2^b* or heterozygous *H-2^{bxu}*.

Antigens and Peptides. Ac-MBP-(1–16) was synthesized by the Keck Foundation protein and peptide core facility at Yale University School of Medicine. The peptide was purified by reverse-phase HPLC and characterized by MS. Chicken ovalbumin (OVA) was purchased from Sigma (A5503).

Flow Cytometry Analysis. The following mAbs were used as first-reagent mAbs in cell surface staining: anti-mouse CD4 conjugated to RED613 (GIBCO); anti-mouse $CD8\alpha$ conjugated to R-phycoerythrin (PE) (GIBCO), anti-mouse $V\beta$ 8.1, 8.2 TCRs (biotin-labeled, Pharmingen), and the clonotypic mAb 19G (3) [purified from culture supernatant on a protein G-Sepharose 4FF column (Pharmacia)]. mAbs Y-3JP, Y-25, and Y-17 were used as hybridoma culture supernatants. Second-step reagents were: fluorescein isothyocyanate (FITC)-conjugated streptavidin (Caltag, South San Francisco, CA) and FITC-conjugated Fc-specific anti-mouse IgG (Sigma). Single-cell suspensions of 1×10^6 thymocytes, spleen cells, or peripheral blood lymphocytes were stained with 50 μl of the first-reagent mAbs at the appropriate dilution in phosphate-buffered saline (PBS) with 1% (vol/vol) fetal calf serum (FCS) and 0.01% sodium azide (staining buffer). After 30 min of

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Abbreviations: TCR, T-cell receptor; OVA, ovalbumin; MHC, major histocompatibility complex; MBP, myelin basic protein; mAb, monoclonal antibody; APC, antigen-presenting cells; FACS, fluorescence-activated cell sorting; V, variable region; Ac-MBP-(1–16), acetylated N-terminal 16-amino acids fragment of MBP.

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incubation on ice, the cells were washed three times with staining buffer, and second-step reagents were added under the same conditions. For two or more color analyses, the cells were incubated with 100 μg of mouse immunoglobulin per ml, and subsequent mAbs were added sequentially with washes in between. The samples were analyzed on a Becton Dickinson FACScan.

Cell Separations. A suspension of $CD4^+$ T cells was prepared by using magnetic beads (Advanced Magnetics, Cambridge, MA). A lymphocyte cell suspension from spleen and lymph node was incubated with mAbs to CD8 (TIB105) and I-A (Y3JP) at appropriate dilutions for 30 min on ice. Cells were then washed, resuspended in medium containing goat anti-mouse IgG- and IgM-coated and goat anti-rat IgG-coated magnetic beads, and incubated on ice on a rotating platform for 30 min. The magnetic beads were then separated by magnet from the purified $CD4^+$ T-cell suspension.

T-Cell Proliferation Assay. $CD4^+$ T cells prepared from lymph nodes and spleen were cultured in microtiter plates (1×10^5 cells per well) with 2×10^5 T cell-depleted and γ-irradiated [2000 rads (20 Gy)] spleen cells and with serial dilutions of Ac-MBP-(1–16). After 48 hr of culture, the cells were pulsed with 1 μCi (37 kBq) of [^{3}H]thymidine and harvested 12 hr later. Incorporation of [^{3}H]thymidine was determined by liquid scintillation counting and presented as mean cpm of triplicate cultures. Standard deviations were <20% of the mean and are not shown.

Cloned T-Cell Lines. The T-cell clone 2R1 was generated by immunizing an *H-2^b*, MBP-specific TCR transgenic mouse in the hind foot pads with 100 μg of OVA in PBS emulsified in complete Freund's adjuvant. Six days later, the mouse was sacrificed, the draining lymph nodes were removed, and a single-cell suspension was prepared. Cells were cultured in bulk by alternative weekly stimulation with OVA presented by $H\text{-}2^b$-expressing APCs (γ-irradiated spleen cells) or Ac-MBP-(1–16) presented by $H\text{-}2^u$-expressing APCs in Click's EHAA medium (GIBCO) containing 10% FCS and 20 units of interleukin 2 per ml. Cells were cloned by limiting dilution and screened for antigen reactivity.

T-Cell Clone Proliferation and mAb Inhibition of Proliferation. Cloned T cells (2×10^4) were cultured with either Ac-MBP-(1–16) or OVA presented by 2×10^5 γ-irradiated spleen cells of mice expressing $I\text{-}A^b$ or $I\text{-}A^u$. After 48 hr of culture, the cells were pulsed with 1 μCi of [^{3}H]thymidine and harvested 12 hr later. Incorporation of [^{3}H]thymidine was determined by liquid scintillation counting in cpm. For inhibition of T-cell-clone proliferation by mAbs, anti-Vβ8.2 TCR (F23.2, purified from hybridoma culture supernatant) and anti-clonotypic mAb 19G were added to the cultures in serial dilutions, and the assay was performed as described above.

Experimental Autoimmune Encephalomyelitis (EAE) Induction and Measurement of Disease. EAE was induced by immunization in the hind foot pads and subcutaneously at the base of the tail with Ac-MBP-(1–16) (200 μg per mouse) in PBS emulsified in complete Freund's adjuvant supplemented with 4 mg of *Mycobacterium tuberculosis* (strain H37Ra) per ml. *Bordatella pertussis* toxin (200 ng per mouse; List Biological Laboratories, Campbell, CA) was injected intravenously in the tail vein at the time of immunization and again 48 hr later. The mice were monitored daily for onset and severity of disease by a "blind" observer and graded according to the following scale: grade 0, no sign of disease; grade 1, limp tail; grade 2, weakness in the hind limbs; grade 3, complete hind limb paralysis; grade 4, hind and front limb paralysis; grade 5, moribund; grade 6, death.

Mixed Lymphocyte Culture. T cell-enriched responder cells were prepared from spleens by incubating a single-cell suspension with anti-MHC class II mAb (Y-3JP) on ice for 30 min. Cells were then washed, resuspended in medium containing magnetic beads coated with goat anti-mouse IgG and IgM, and incubated on ice on a rotating platform for 30 min. The magnetic beads were separated by magnet from the target cell suspension. The responder cells were then titrated in serial dilutions starting at 2×10^5 cells per well. Stimulator cells (γ-irradiated spleen cells) were added to the responder cells at a constant cell number, 5×10^5 cells per well. After 72 hr of culture, the cells were pulsed with 1 μCi of [^{3}H]thymidine and harvested 12 hr later. Incorporation of [^{3}H]thymidine was determined by liquid scintillation counting in cpm.

RESULTS

Strong Positive Selection of an Autoreactive TCR in TCR Transgenic Mice. These studies use a newly generated transgenic mouse line carrying the rearranged TCR α and β chain genes of a cloned line of T cells specific for Ac-MBP-(1–16) (5) bound and presented by $I\text{-}A^u$ on the cell surface. This cloned T-cell line, called clone 19, can produce EAE when transferred to irradiated syngeneic mice (3). When the transgene is bred to mice that express $I\text{-}A^u$, the developing T cells in the thymus are strongly positively selected, being either $CD4^+$ $CD8^-$ and expressing high levels of the TCR encoded by the parent cloned line [as detected by antibody 19G specific for the clonotypic receptor of clone 19 (3)] or $CD4^+$ $CD8^+$ and expressing intermediate levels of this TCR (Fig. 1 *Middle*). In the peripheral lymphoid tissues, mainly $CD4^+$ cells but few $CD8^+$ T cells are seen in mice that express $I\text{-}A^u$, and all of the $CD4^+$ T cells express high levels of the transgene-encoded TCR (Fig. 1 *Middle*, see also Fig. 3 *Upper*). By contrast, when the transgene is bred to mice that express $I\text{-}A^b$ but not $I\text{-}A^u$, the thymus contains mainly $CD4^+$ $CD8^+$ cells with lower levels of the transgene-encoded TCR, while the peripheral lymphoid organs have both $CD4^+$ and $CD8^+$ T cells that express reduced levels of the clonotypic receptor (Fig. 1 *Bottom*). Thus, these mice show a strong positive selection specific for $I\text{-}A^u$ as expected from earlier studies of this type (6, 7). Moreover, despite expressing a TCR that is specific for a self-peptide bound to self-MHC, they are not self-tolerant, since exposure to the peptide Ac-MBP-(1–16) induces a strong T-cell proliferative response (Fig. 2 *Left*), and immunization with MBP induces a rapidly fatal course of EAE (Fig. 2 *Right*) that is not seen in transgene-negative littermates. Thus, these results are

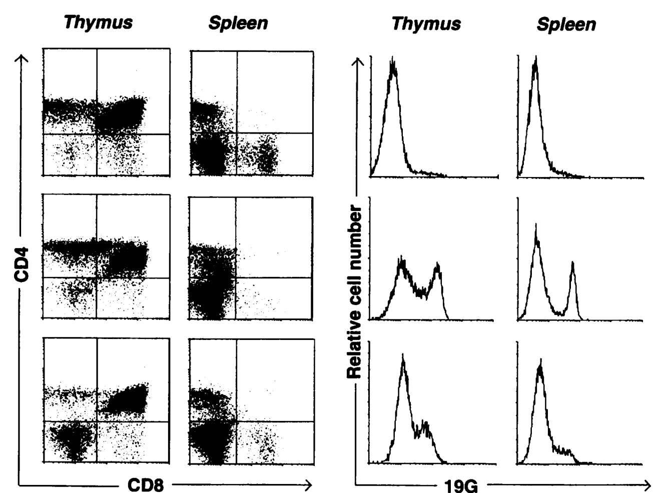

FIG. 1. Positive selection of lymphocytes bearing the transgene-encoded MBP-specific TCR. Thymocytes and spleen cells were prepared from nontransgenic littermates (*Top*), and the MBP-specific TCR-expressing transgenic mice on selective (*H-2^u*) (*Middle*) and nonselective (*H-2^b*) (*Bottom*) MHC haplotypes. Cells (1×10^6) were stained with antibodies specific for CD4 (vertical axis), CD8 (horizontal axis), and the transgenic MBP-specific TCR (19G) (histograms) and then were analyzed by FACS.

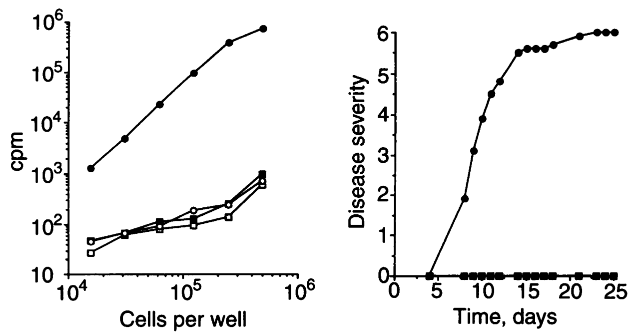

FIG. 2. The MBP-specific TCR-expressing transgenic mice are not self-tolerant. (*Left*) Exposure in the peptide Ac-MBP-(1–16) *in vitro* induces a strong proliferative response in transgenic mice but not in transgene-negative littermates. Spleen cells from transgenic and nontransgenic littermates were cultured at various concentrations with or without the addition of Ac-MBP-(1–16) (10 μg/ml). The proliferative response was assessed 3 days later by measuring the incorporation of [^{3}H]thymidine. ○, Transgenic cells; ●, transgenic cells + 10 μg of Ac-MBP-(1–16) per ml; □, nontransgenic cells; ■, nontransgenic cells + 10 μg of Ac-MBP-(1–16) per ml. (*Right*) Immunization with Ac-MBP-(1–16) induces a rapidly fatal course of EAE that is not seen in transgene-negative littermates. Transgenic (●) and nontransgenic (■) littermates were immunized with Ac-MBP-(1–16) in complete Freund's adjuvant and *B. pertussis* toxin. The mice were monitored for onset and severity of disease.

comparable to and confirm earlier studies of this type (6, 7), including a previous analysis of mice transgenic for a receptor of similar specificity (8).

Development of T Cells Expressing Two Different TCRs in TCR Transgenic Mice Lacking the Selecting MHC Molecules. Although *H-2^u* mice show strong positive selection of the clone 19 TCR, while the development of CD4$^+$ T cells in *H-2^b* TCR transgenic mice is severely retarded (data not shown), we have also observed, as have others (9), that developing T cells that do not encounter the MHC molecule required for positive selection can undergo further rearrangement of *Vα* gene segments to the joining region α chain (Jα) (10), and that some of these will generate *Vα* exons that encode a TCR specific for self-MHC, which allows them to undergo further development in the absence of I-A^u (Fig. 1 *Bottom*). We have analyzed these T cells for the expression and function of the TCR generated from the transgene-encoded β chain and endogenous α chains. These CD4$^+$ T cells bearing two different TCRs arise slowly in TCR transgenic mice bearing I-A^b but are not seen in mice expressing I-A^u. This is shown by the lower level of staining with the clonotypic antibody 19G than with the Vβ8.2-specific antibody on CD4$^+$ T cells from I-A^b-expressing mice but not on CD4$^+$ T cells from I-A^u-expressing mice (Fig. 3 *Upper*). That CD4$^+$ T cells from I-A^b bearing mice express a functional transgene-encoded TCR is shown by their ability to respond to Ac-MBP-(1–16) when it is presented by cells bearing I-A^u but not when it is presented by H-2^b-bearing cells matched at the MHC with the T-cell donor (Fig. 3 *Lower*). As these CD4$^+$ T cells express the transgene-encoded β chain at normal levels but have reduced levels of the clonotypic receptor, they must have rearranged an endogenous *Vα* gene segment that pairs with some of the transgene-encoded β chain to form an I-A^b-restricted TCR.

Expression by One T Cell of Two TCR Specific for Different Peptide–MHC Complexes. Earlier studies have shown that TCR transgenic mice can express more than one TCR (1), and a recent study in humans showed that about one T cell in three expresses two different *Vα* gene segments (2). Moreover, in these latter studies of human T cells, it was shown that individual cloned T-cell lines bearing two TCRs could be triggered by antibodies specific for either TCR (2). However,

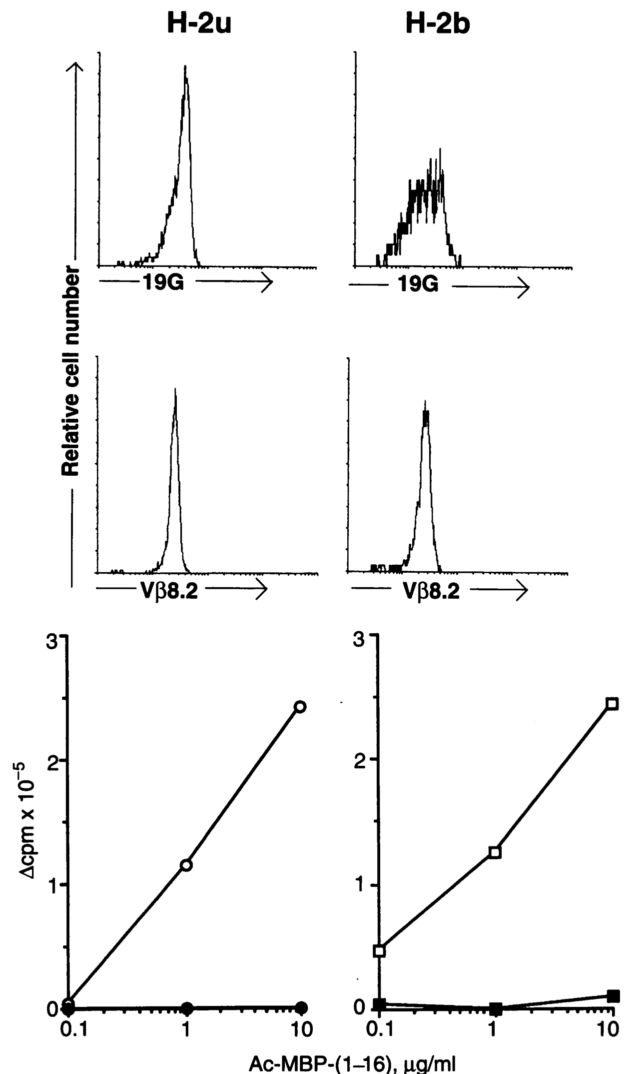

FIG. 3. Development of the MBP-specific TCR-expressing transgenic T cells in selecting and nonselecting MHC haplotypes. (*Upper*) FACS analysis of staining of CD4$^+$ T cells from mice on a selecting haplotype (*I-A^u*) and nonselecting haplotype (*I-A^b*) with the clonotypic antibody 19G and anti-Vβ8.2 antibody. Peripheral blood lymphocytes were prepared from 10-week-old MBP-specific TCR-expressing transgenic mice on selective (*H-2^u*) and nonselective (*H-2^b*) MHC haplotypes. Cells (1 × 10^6) were stained with mAbs specific for CD4, CD8, and either the transgenic TCR 19G or Vβ8.2 and analyzed by FACS. The staining intensity of 19G and Vβ8.2 of the CD4$^+$ population was analyzed. (*Lower Left*) CD4$^+$ T cells prepared from mice on a selecting background (*I-A^u*) respond to the peptide Ac-MBP-(1–16) only when it is presented by APC that are *I-A^u* (○) and not by other haplotypes—e.g., *I-A^b* (●). (*Lower Right*) CD4$^+$ T cells isolated from MBP-expressing TCR transgenic mice on a nonselecting background (*I-A^b*) respond to MBP Ac1-16 only when it is presented by *I-A^u* APCs (□) and not when it is presented by cells of their own MHC haplotype (■). CD4$^+$ T cells were prepared from spleens by Magnetic bead cell separation; 1 × 10^5 CD4$^+$ T cells were cultured with various concentrations of MBP Ac1-16 and 2 × 10^5 T-cell depleted and γ-irradiated spleen cells of different MHC haplotypes as APCs. Proliferation was assessed as [^{3}H]thymidine incorporation after 3 days of culture. Data is presented as Δcpm.

the specificity of these two TCRs for antigen and more importantly for MHC has not been determined. Since the T cells that arise in our TCR transgenic mice that lack I-A^u respond nevertheless to Ac-MBP-(1–16) presented by I-A^u spleen cells, we knew the specificity of at least one of the TCRs expressed on these cells. Therefore, we immunized *H-2^b* TCR

transgenic mice with OVA and raised T-cell lines by alternate stimulation with OVA presented by I-A^b-bearing spleen cells and with Ac-MBP-(1–16) presented by I-A^u-bearing spleen cells. The resulting T-cell line, and clones derived from it, responded to both stimuli (Fig. 4 *Upper*). These T cells, like the T cells in the donor mouse, bore normal levels of the transgene-encoded $V\beta$ domain but reduced levels of the transgene-encoded clonotype, indicating that a second $V\alpha$ gene was rearranged and expressed. That this second rearrangement accounted for the recognition of OVA presented by I-A^b-bearing cells is shown by inhibiting the response of cloned, dual reactive T cells to both antigens with anti-Vβ8.2 antibody, while the clonotypic antibody blocked only the response to Ac-MBP-(1–16) presented by I-A^u-bearing cells (Fig. 4 *Lower*).

Alloreactivity Arises Preferentially in Mice That Cannot Positively Select the Nonalloreactive Transgene-Encoded TCR. The TCR used to prepare these TCR transgenic mice did not show strong responses to any known non-self-MHC protein. In keeping with this, the T cells from young, TCR transgene-positive *H-2^u* mice do not make responses to nonself MHC-bearing stimulator cells or to superantigens of PL/J mice that are stimulatory to B10.PL T cells. However, T cells from *H-2^b* TCR transgenic mice show significant responses to nonself MHC molecules (Fig. 5). These cells must have at least two different TCRs, as the transgene-encoded TCR expressed by these cells lacks alloreactivity. This experiment shows that self-MHC recognition by the first TCR expressed by a developing T cell prevents its acquisition of a second TCR, strongly supporting the idea that the same T cell is unlikely to express two self-MHC-restricted TCRs. It leaves open the origin of alloreactivity in normal T cells, since in this case alloreactivity could result from a TCR restricted to *H-2^b* that was positively selected in these mice or from a third endogenous TCR that is neither transgene-encoded nor selected on *H-2^b*.

DISCUSSION

These studies show that T cells violate the rule of allelic exclusion of receptors and can have dual specificity for antigen and MHC, confirming earlier studies (1, 2) and extending them by documenting the antigen and MHC specificity of both TCRs. Earlier studies of normal T cells in humans had suggested that one T cell in three bears receptors encoded by two different $V\alpha$ gene segments (2). If one calculates the proportions of developing thymocytes expected to carry one or two different $V\alpha$ gene segments, one finds that four should have one TCR for every one that has two. This statement is based on the assumption that rearrangement of $V\alpha$ to $J\alpha$ is a random process with one chance in three to be in frame on each chromosome. If this is so, then one cell in nine should have two in-frame $V\alpha$-$J\alpha$ joins, four of nine should have one, and four of nine should have none, provided positive selection is a sufficiently rare event to allow rearrangement to come to equilibrium, which takes five rearrangement events if rearrangement is sequential, and two if it occurs simultaneously on both chromosomes. Thus, one would expect the ratio of thymocytes with two TCRs to those with one to be 4:1. Moreover, if positive selection is an infrequent event, T cells with two TCRs will be roughly twice as likely as those expressing one TCR to undergo positive selection, an event that inhibits further TCR gene rearrangement (9). Therefore, in the periphery, one T cell in three should have two different TCRs, which is precisely the result obtained by Padovan *et al.* (2).

Padovan *et al.* (2) speculated that expression of two different TCRs might contribute to autoimmune responses and to alloreactivity. Although our data on the development of T cells bearing two TCRs are consistent with those of Padovan *et al.* (2), our data do not provide support for these speculations. Using an autoreactive TCR, we find that positive selection for

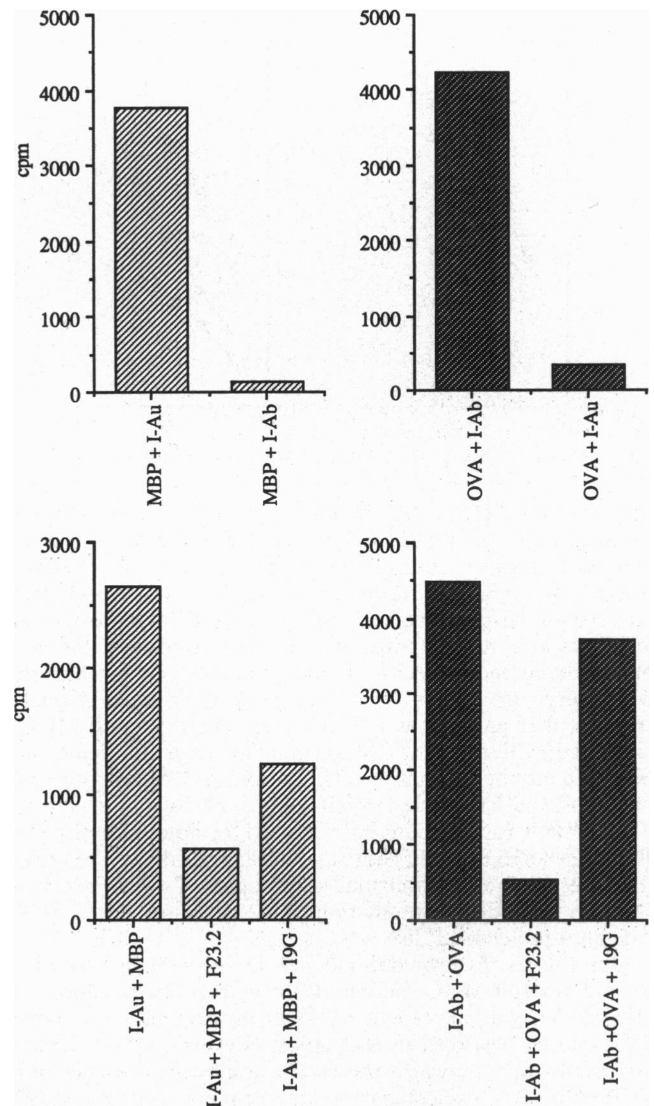

FIG. 4. T-cell clone 2R1 responds to both Ac-MBP-(1–16) presented by I-A^u-bearing cells and OVA presented by I-A^b-bearing cells. (*Upper*) T-cell clone 2R1 (2×10^4 cells), cultured in an *in vitro* assay, proliferates in response to Ac-MBP-(1–16) at 10 μg/ml only when it is presented by (2×10^5) I-A^u-bearing and not by I-A^b-bearing APCs (*Upper Left*) and in response to OVA at 300 μg/ml only when presented by (2×10^5) I-A^b-bearing and not by I-A^u-bearing APCs (*Upper Right*). (*Lower*) Anti-Vβ8.2 (F23.2) antibody blocks both responses, whereas clonotypic antibody (19G) only blocks response to Ac-MBP-(1–16). The T-cell clone 2R1 (1×10^4 cells) was cultured in an *in vitro* assay with Ac-MBP-(1–16) at 10 μg/ml presented by (1×10^5) I-A^u-bearing spleen cells (*Lower Left*) or OVA at 300 μg/ml presented by I-A^b-bearing spleen cells (*Lower Right*) in the presence of F23.2 or 19G at 10 μg/ml.

self-MHC recognition is sufficiently efficient to virtually preclude the expression of other TCRs. Indeed, our data show that the possession of two TCRs is not necessary for the development of T cells capable of inducing autoimmune disease. Moreover, our data suggest that the expression of one self-MHC-restricted TCR is sufficient to prevent expression of other TCRs on the same cells. Further, if a T cell with a TCR reactive to a ubiquitous self peptide were to arise, it would be eliminated, whether it expressed one or two TCRs. Finally, our studies confirm those of Goverman *et al.* (8) by showing that autoreactive T cells bearing a single TCR arise normally and can even be abundant. More importantly, we have shown that when a T cell does arise bearing two MHC-restricted TCRs,

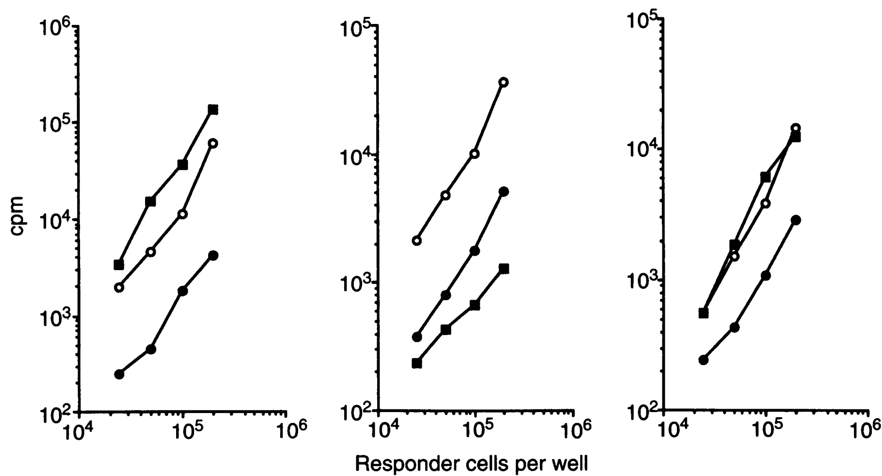

FIG. 5. T cells from H-2^u TCR transgenic mice do not respond to nonself-MHC-bearing stimulator cells, whereas T cells from H-2^b TCR transgenic mice do. Various numbers of responder cells were tested for response to irradiated spleen cells as stimulator cells of various MHC haplotypes in an *in vitro* mixed lymphocyte assay. Cultures were pulsed with [^{3}H]thymidine after 72 hr and harvested 12 hr later, and the response was assessed as incorporation of [^{3}H]thymidine. ○, Nontransgenic littermate responder cells; ●, H-2^u (on B10.PL background) transgene-positive mouse cells; and ■, H-2^b transgene-positive mouse cells. (*Left*) PL/J stimulator cells (H-2^u, *Mls c*). (*Center*) C57BL/6 (H-2^b, *Mls b*) stimulator cells. (*Right*) B10.A(4R) (H-2^k, *Mls b*) stimulator cells.

only one of the TCRs is restricted to self-MHC. Thus, while the finding of T cells with two functional TCRs (1, 2) may seem to violate a primary tenet of the clonal selection hypothesis—namely, that cells bear receptors of a single specificity—in fact only one of the TCRs we have observed is self-MHC-restricted and thus able to recognize antigen *in vivo*, making the cell operationally monospecific. Finally, the lack of alloreactivity we observe in T cells from transgenic H-2^u mice strongly suggests that as soon as a T cell does express a self-MHC-restricted TCR, further TCR rearrangements are inhibited and the cell continues its maturation. Therefore, T cells expressing two TCRs, both restricted to self-MHC molecules, are likely to be extremely rare and are not required for autoimmunity. To show that such cells contribute to autoimmune disease requires the direct demonstration that autoreactive T cells have two specificities and that stimulation of the nonautoreactive TCR triggered the clinical disease.

Our studies do provide evidence for a possible role of a second nonself-MHC-specific TCR in the phenomenon of alloreactivity, as alloreactive T cells bearing the transgenic TCR can be observed in H-2^b mice. However, this system is artificially forced towards this conclusion by the presence of a TCR that is not selectable in the recipient. Moreover, the transgene-encoded TCR does not provide for the alloreactivity of the T cells that bear it; alloreactivity comes instead from receptors selected on the H-2^b background in this case, and these are likely to be both self-MHC-restricted and alloreactive, as is true of several other TCR that have been shown to have similar dual specificity. Studies using clonotypic antibodies to block a given TCR (11, 12) as well as transfection data (13–16) have yet to reveal cells that have two TCRs that mediate recognition of antigen and self-MHC separately from recognition of non-self-MHC. Indeed, it appears from these studies that, in each case, alloreactivity involves cross-reactions of TCR specific for a foreign antigenic peptide bound to self-MHC molecules. However, further analysis of the T cells that express two TCRs may well reveal examples of alloreactivity due to a TCR that is not self-MHC-restricted. A possible example of this are the T cells reported by Hunig and Bevan (17), which responded to different minor H antigens bound to different MHC molecules. Although originally thought to reflect dual specificity of one receptor, these cells may in fact reflect the action of two TCRs, analogous to the results reported here.

In conclusion, we have shown using TCR transgenic mice that T cells can express two different peptide—MHC-specific TCRs through expression of two different $V\alpha$ genes. However, our data suggest that only one of these TCRs is likely to be self-MHC-specific, and we conclude from this that it is unlikely that a T cell with an autoreactive TCR will be activated by foreign peptides bound to self-MHC molecules acting on a different TCR. Rather, our data show that T cells expressing a single, autoreactive TCR can lead to autoimmune disease. Moreover, most data suggest that a single TCR accounts for recognition of antigen–self-MHC as well as nonself-MHC in responses to allogeneic stimulators. Thus, while T cells can express more than one TCR, it is not clear that this represents a violation of the principle that cells have a single functional specificity, as only T cells with two self-MHC-restricted TCRs would be operationally bispecific, and we have not observed such cells in this study.

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