The Stimulation of Low-Affinity, Nontolerized Clones by Heteroclitic Antigen Analogues Causes the Breaking of Tolerance Established to an Immunodominant T Cell Epitope

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Summary

H-2K mice injected, intravenously in saline or intraperitoneally in incomplete Freund's adjuvant, with large quantities of the immunodominant I-E^k–restricted epitope from moth cytochrome c (MCC) 88–103 fail to respond to subsequent immunization with this epitope when administered in complete Freund's adjuvant. This state of tolerance can be broken by immunization with certain MCC 88–103 analogues that are heteroclitic antigens as assessed on representative MCC 88–103 specific T cell clones. In this paper, the mechanism of breaking tolerance by heteroclitic antigens was investigated. The following observations were made: (a) T cell hybridomas derived from tolerance-broken animals required higher concentrations of MCC 88–103 to be stimulated than hybridomas derived from normal immune animals, suggesting that they have T cell receptors (TCRs) of lower affinity; (b) in contrast to normal immune animals whose MCC-specific TCRs are typically $V\beta3^+/V\alpha11^+$, none of the hybridomas derived from tolerance-broken animals expressed Vβ3, although they were all $V\alpha11^+$. Also, the Vβ complementarity determining region 3 (CDR3) regions from the tolerance-broken animals did not contain the canonical structure and length characteristics of the normal MCC 88–103 immune repertoire; and (c) adoptive transfer and tolerization of MCC-specific $V\beta3^+/V\alpha11^+$ transgenic T cells followed by immunization with heteroclitic antigen failed to terminate the state of tolerance. Collectively, these data strongly suggest that the mechanism involved in breaking tolerance in this system is the stimulation of nontolerized, low-affinity clones, rather than reversal of anergy. Further support for this mechanism was the finding that after activation, T cells apparently have a lowered threshold with respect to the affinity of interaction with antigen required for stimulation.

Key words: molecular mimicry • cytochrome c • T cell repertoire • tolerance

The definition of the mechanisms by which tolerance is established to an antigen, and once established, the mechanisms by which tolerance can be terminated, are fundamental to understanding the generation of autoimmune responsiveness. Many examples exist that document the potential of peptides structurally related to self-antigens to induce an immune response that is cross-reactive with the self-epitope (1–7). However, the mechanism by which tolerance is broken in most instances is poorly understood.

T cell tolerance to self-determinants may be mediated by a variety of mechanisms that act centrally on developing T cells in the thymus or on mature T cells in the periphery. Clonal deletion appears to be the dominant mechanism by which central tolerance is achieved to self-proteins that are presented within the thymus (8–10). However, this process may be incomplete or the self-antigen may not be presented within the thymus, resulting in the appearance of potentially autoreactive T cells in the periphery. Tolerance in the periphery may be achieved by a variety of mechanisms, including clonal deletion, anergy, and ignorance (in which the epitope is not presented in sufficient quantity by a professional APC to achieve T cell activation). These direct and cell-intrinsic mechanisms are further complicated by regulatory mechanisms such as suppression and immune deviation (11–20). Many of the previous studies of peripheral self-tolerance have been difficult to interpret because of not having suitable nontolerized control animals with which to compare the results obtained in self-antigen expressing animals. The generation of self-antigen knockouts and the use of foreign antigen transgenic mice have made

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the appropriate studies more feasible than previously. However, even in these systems, since both central and peripheral mechanisms may be operative, a detailed mechanistic study of peripheral tolerance is difficult. In contrast, studies of peripheral tolerance to foreign antigens have established that both cell death and anergy can be involved in the generation of tolerance under circumstances where the fate of tolerized transgenic T cells can be followed with anticlonotypic antibodies (21–25).

We have initiated the study of a model of adult tolerance to a foreign immunodominant epitope and have analyzed the breaking of that tolerance by structurally related, cross-reactive antigens so that we could gain information on the mechanism by which peripheral T cell tolerance is established and on how molecular mimicry may operate in the breaking of peripheral tolerance. Using the I-E^k–restricted epitope moth cytochrome c 88–103 (pMCC)[1] as the tolerogen, it was found that certain single amino acid–substituted analogues of the tolerogen were capable of breaking tolerance to the antigen (26). The successful analogues were all heteroclitic antigens, in that they stimulated a representative pMCC-specific T cell clone at a lower concentration than that required for the cognate antigen. Immune deviation was excluded as a possible mechanism in the establishment and the termination of the tolerant state in this system. In this study, two other possible mechanisms for breaking tolerance were evaluated: (a) the stimulation of a subset of T cells that, because they possessed too low an affinity for the tolerogen, had escaped tolerance induction; and (b) the reactivation of previously anergized T cells. The accumulated data strongly support the first mechanism, namely the activation of T cells that were of too low affinity to be tolerized, but once stimulated by the heteroclitic antigen became responsive to subsequent stimulation with the tolerizing epitope.

Materials and Methods

Animals and Cell Lines. Female B10.A mice (The Jackson Laboratory) were used at 8–10 wk of age. Invariant chain–deficient B10.BR mice were provided by Dr. D. Mathis (IGBMC, Strasbourg, France) and used at 6–12 wk of age. AND and AD10 transgenic mice containing pigeon cytochrome c (PCC)–specific TCRs were provided by Dr. S. Hedrick (University of California, San Diego, CA), as was the cytochrome c–specific T cell clone, AD10. The B cell lymphoma CH27, provided by Dr. G. Hough-ton (University of North Carolina, Chapel Hill, NC), was used as APC in some experiments. For IL-2 determinations, the IL-2–dependent T cell line CTLL-2 was used. BW5147$\alpha^-\beta^-$ cell line was used as fusion partner in the production of T cell hybridomas.

Peptide Synthesis. pMCC (ANERADLIAYLKQATK) and the heteroclitic analogue (pMCC-A; AAAAAAAIAYAKQATK) were synthesized on a Rainin Symphony synthesizer (Peptide Technologies) as described previously (27). Peptides were purified by reverse-phase HPLC to >95% purity. Identity of the peptides was substantiated by mass spectrometry.

Tolerance Induction and Immunization. Tolerance induction and immunization were performed as described previously (26). In brief, to induce tolerance, B10.A mice were injected intraperitoneally with 300 μg pMCC in IFA (Pierce Chemical Co.). 10 d later, tolerized mice were immunized with 20 μg pMCC or the heteroclitic antigen analogue, pMCC-A, in CFA (Difco) subcutaneously in the base of the tail. As controls, normal mice were immunized with 20 μg pMCC or pMCC-A in CFA. 10 d after immunization, draining lymph nodes were removed and single cell suspensions were made and used for proliferation and cytokine assays or to establish short-term T cell lines and hybridomas.

Proliferation Assays. Cells from draining lymph nodes (paraaortic and inguinal) were plated at 2.5×10^5 cells/well along with 2.5×10^5 irradiated (3,000 rad) syngeneic splenocytes and stimulated with the pMCC or pMCC-A peptide as indicated. After 72 h, the cultures were pulsed for an additional 18 h with 1 μCi of [^{3}H]thymidine and analyzed by beta-plate scintigraphy. In experiments to analyze relative immunogenicity of MCC analogues, proliferation assays were performed using the AD10 clone, as described previously (27).

Cytokine Assays. For cytokine analysis, supernatants from the cultures that had been established to measure the proliferative response were removed after 24 h for IL-2 measurement, after 48 h for IL-4 and IL-10, or after 72 h for IFN-γ. IL-2 was measured by bioassay using the CTLL-2 cell line. IL-4, IL-10, and IFN-γ were assayed by ELISA according to the instructions provided by the manufacturer of the reagents (PharMingen). The sensitivities of the ELISA assays were as follows: IFN-γ, 100 pg/ml; IL-4, 50 pg/ml; and IL-10, 50 pg/ml.

I-E^k Binding Assay. Peptides were analyzed for their ability to bind to purified I-E^k molecules as described previously (28). The binding capacity is reported as the concentration of peptide required to obtain 50% inhibition (IC_{50}) of binding of the radiolabeled ligand.

Adoptive Transfer of MCC-specific Transgenic T Cells. $CD4^+$ T cells were purified from the lymph node and spleen of pMCC-specific TCR transgenic mice (AND) by collecting nonadherent cells from a nylon wool column, followed by complement treatment of the cells that had previously been incubated with antibodies to CD8 (3.155), heat-stable antigen (JIID), class II MHC (M5/114 and CA-4.A12), macrophages (M1/70), and dendritic cells (33D1), and subsequently cross-linked with mouse anti–rat kappa antibody (MAR18.5). The percentage of $CD4^+$ cells that were $V\alpha11^+V\beta3^+$ T cells was determined by flow cytometry. This information was then used to calculate the total number of cells that had to be injected to achieve 2.5×10^6 $V\alpha11^+V\beta3^+$ T cells/injection. Mice were injected intravenously with this number of cells in a vol of 0.4 ml of HBSS (GIBCO BRL) (21). Recipient mice were unirradiated, invariant chain–deficient B10.Br mice. 3 d after cell transfer, the animals were injected intravenously with 300 μg of pMCC in a vol of 250 μl to induce tolerance. 10 d later, tolerized and nontolerized mice were immunized with 20 μg of pMCC or pMCC-A in CFA subcutaneously. After an additional 10 d, draining lymph nodes were removed and single cell suspensions were used for in vitro stimulation and cytokine analysis.

T Cell Hybridomas. Draining lymph node cells from immunized or tolerance-broken mice were restimulated in vitro with 0.5 μM pMCC or pMCC-A for 2 d and expanded with IL-2 (20 U/ml) for an additional 2 d. The lymphoblasts thus generated were fused with the BW5147$\alpha^-\beta^-$ cell line at ~1:3 ratio, and hybridomas were selected in HAT medium and distributed in 96-well plates at $\sim10^4$ cells/well (29). Wells were screened for

[1]*Abbreviations used in this paper:* BI, beef insulin; MCC, moth cytochrome c; pMCC, MCC 88–103; pMCC-A, pMCC heteroclitic polyalanine analogue.

Table I. *MHC Binding and Antigenicity of pMCC and pMCC-A*

	Sequence	I-E^k binding*	Antigenicity‡
		nM	*nM*
pMCC	ANERADLIAYLKQATK	31	2.8
pMCC-A	AAAAAAAIAYAKQATK	36	0.3

*Concentration of peptide required to inhibit by 50% the binding of a standard radioiodinated peptide to soluble I-E^k.
‡Concentration of peptide required to achieve 40% of maximum proliferation of the pMCC-specific T cell clone, AD10.

reactivity to pMCC or pMCC-A by measuring IL-2 production. The positive wells were cloned at a concentration of 0.3 cells/well, and clones that produced IL-2 in response to antigen were expanded for further analysis.

Flow Cytometry. Cells were incubated on ice with anti-CD3–FITC, anti-Vα11–FITC, anti-Vβ3–PE (PharMingen), or anti-CD4–PE (Becton Dickinson) for 1 h and washed several times. Live cells were gated by forward and side scatter and analyzed by flow cytometry on a FACScan™ equipped with CELLQuest™ software (Becton Dickinson).

DNA Sequence Analysis. Total RNA was isolated from T cell hybridomas using an RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. Oligo dT–primed cDNA was synthesized using a cDNA Cycle kit (Invitrogen Corp.) following the manufacturer's protocol and then amplified by PCR for sequence analysis. The predicted size fragments from PCR amplification were further purified with a QIAquick Gel Extraction kit (QIAGEN) and sequenced using the Taq Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems) on an Applied Biosystems 310 DNA Sequencer. The PCR and sequencing primers used were as follows: for α chain PCR: Cα, AAG TCG GTG AAC AGG CAG AG; for Vα11, TCA GGA ACA AAG GAG AAT GG; for α chain sequencing: Cα, AAC TGG TAC ACA GCA GGT TC; for β chain PCR primers: Cβ, AGC ACA CGA GGG TAG CCT T (30); for β-chain sequencing: GGA GTC ACA TTT CTC AGA TCC A. A set of Vβ primers was used for PCR as reported previously (31).

Results

In a previous study to determine whether cross-reactive antigen analogues could break tolerance induced to the immunodominant T cell epitope from MCC, pMCC, it was found that certain heteroclitic antigens (as defined by their heightened antigenicity for a representative T cell clone) were capable of breaking tolerance (26). Specifically, substitutions at the minor MHC contact residue L98 were effective in this regard. In this study, we have used a pMCC analogue with an L98A substitution that also had residues 88–94, which are outside the MHC peptide binding cleft, substituted with alanines. This poly-A MCC analogue (pMCC-A) had a similar binding capacity to I-E^k as the native peptide, but was able to stimulate MCC-specific clones ~10-fold more efficiently than the native pMCC peptide (Table I). The ability of this analogue to break tolerance to pMCC is shown in Fig. 1. Tolerized animals had a markedly reduced capacity to proliferate in response to stimulation with pMCC compared with the response of immunized controls (Fig. 1 A) and did not synthesize detectable amounts of IFN-γ (Fig. 1 B) or other cytokines such as IL-2, IL-4, or IL-10 (data not shown). In contrast, mice that were tolerized to pMCC and then immunized with the heteroclitic analogue, pMCC-A, mounted vigorous proliferative and cytokine responses, albeit requiring 10–100-fold more antigen than the normal immunized controls to achieve comparable responses.

Because of reports that claim administration of antigen intraperitoneally in IFA leads to sequestration of the immune response in the spleen rather than tolerance induction, studies were carried out to evaluate this possibility. Cytokine analysis of spleen and draining lymph nodes after pMCC administration intraperitoneally in IFA followed by pMCC immunization subcutaneously in CFA indicated

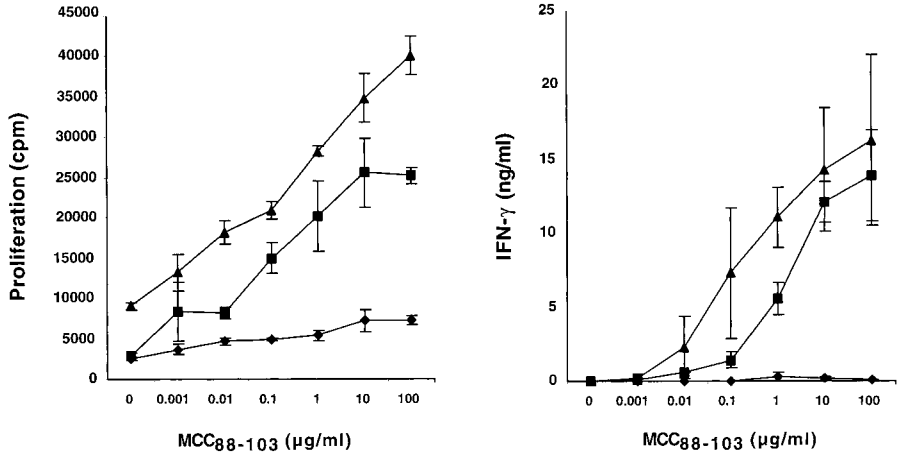

Figure 1. Capacity of a heteroclitic antigen analogue to terminate tolerance. The heteroclitic antigen pMCC-A and the cognate antigen pMCC were used to immunize mice that had been previously tolerized to pMCC. Lymphoid cells from nontolerized, pMCC-immunized mice (▲), tolerized and pMCC-immunized mice (◆), and tolerized and pMCC-A–immunized mice (■) were analyzed for their proliferative activity and production of IFN-γ after in vitro stimulation with the tolerogen, pMCC.

that tolerance was evident in both lymph node and spleen, with undetectable quantities of IFN-γ and IL-4 found in culture supernatants of both organs. Enzyme-linked immunospot (ELISPOT) analysis also indicated tolerance induction, although less dramatic than the supernatant analysis. IFN-γ–producing cells predominated in normal immune lymph node and spleen (600 and 500 cells/10^6), respectively, with a very small IL-4–producing population (<20 cells/10^6). In pMCC-tolerized and immunized animals, there was a 60 and 75% reduction in IFN-γ–producing cells in both spleen and lymph node, with no change or a slight increase in IL-4–producing cells (24 cells/10^6 in spleen). Thus, with respect to proliferation and the major cytokine, IFN-γ, tolerance was clearly established by the protocol used, although the small number of IL-4–producing cells generated was apparently not affected by the tolerance-inducing protocol.

Clonal Analysis of T Cells Involved in Termination of Tolerance. The results shown in Fig. 1 and those reported previously (26) suggest that the T cells involved in the breaking of tolerance have a lower avidity for antigen compared with normal immune T cells, as evidenced by the shift in the antigen dose–response profile. To study in greater detail the functional activity of the T cells involved in the breaking of tolerance to pMCC, and in order to analyze the structure of their TCRs, a series of T cell hybridomas was made from animals whose tolerance to pMCC was broken by immunization with pMCC-A. As controls, hybridomas were also derived from normal animals immunized with either pMCC or pMCC-A. The response of representative hybridomas derived from normal or tolerance-broken animals to stimulation with pMCC is shown in Fig. 2. The two hybridomas derived from pMCC or pMCC-A normal immune animals behaved similarly to stimulation with pMCC. In contrast, and similar to the data obtained with the bulk T cell response, the hybridoma derived from the tolerance-broken group required ~10-fold more pMCC to give an IL-2 response comparable to that of the hybridomas derived from the normal immunized animals. The decreased antigen responsiveness of the hybridomas derived from tolerance-broken animals was not due to a decrease in surface expression of either the TCR or the CD4 coreceptor (Fig. 2, B and C; and data not shown).

Fig. 3 summarizes the antigen response data from all hybridomas that reacted to both pMCC and pMCC-A, as a plot of the quantity of pMCC versus pMCC-A required to generate an IL-2 response in each hybridoma analyzed. Several conclusions can be derived from the data shown in Fig. 3. (a) All but one of the hybridomas derived from the pMCC-immunized group responded to lower concentrations of pMCC-A than pMCC, as evidenced by a location on the graph below the diagonal. This indicates that the heteroclitic nature of pMCC-A, originally described with a single T cell clone, is generalizable to the majority of the pMCC-specific T cell repertoire. (b) The hybridomas in the pMCC tolerance-broken group, in general, required higher concentrations of pMCC to stimulate an IL-2 response than the hybridomas from either the pMCC or

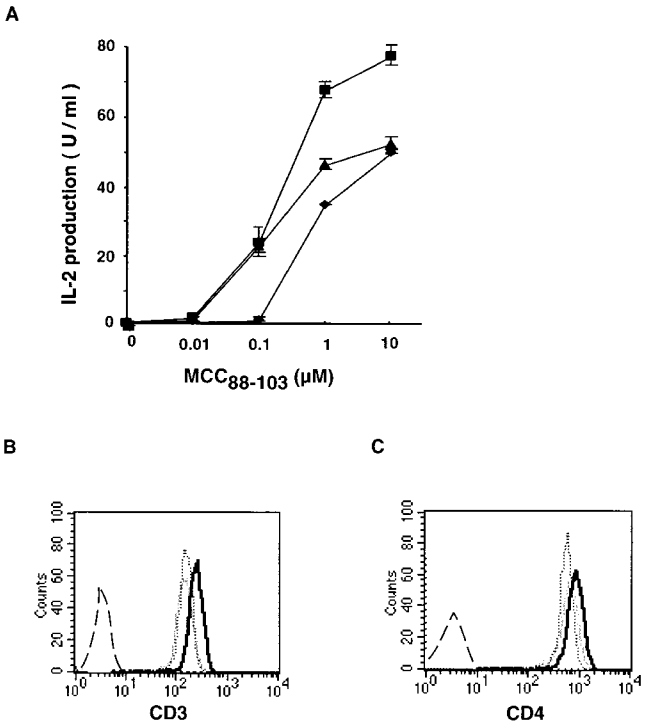

Figure 2. Function and receptor analysis of T cell hybridomas derived from normal immune and tolerance-broken animals. (A) T cell hybridomas derived from mice which were pMCC (▲) or pMCC-A (■) immunized, or tolerized to pMCC and immunized with pMCC-A (◆) were analyzed for their capacity to make IL-2 in response to varying doses of pMCC. (B and C) T cell hybridomas from pMCC-immunized mice (light line), pMCC-A–immunized mice (dotted line), and tolerance-broken mice (bold line) were analyzed for expression of CD3 (B) and CD4 (C). Dashed line indicates negative control staining.

pMCC-A normal immune groups. Thus, the hybridomas from the tolerance-broken group required an average of 0.88 μM of pMCC to stimulate an IL-2 response, whereas the pMCC immune group required 0.01 μM, and the pMCC-A immune group required 0.07 μM pMCC to stimulate a response. (c) In contrast to the greater antigen requirement of the tolerance-broken group for pMCC, the response to pMCC-A was similar for all three groups. In addition to the cross-reactive hybridomas shown in Fig. 3, a small number of hybridomas from pMCC or pMCC-A immune animals were found to be non–cross-reactive (1 of 10 in the pMCC immune group failed to respond to pMCC-A, and 3 of 14 of the pMCC-A group failed to respond to pMCC; data not shown).

Structural Characteristics of the TCRs from T Cells Involved in the Breaking of Tolerance to pMCC. The antigen dose–response data for pMCC indicated that the hybridomas from the tolerance-broken group had a lower avidity for pMCC than hybridomas from normal immune animals. To determine if this functional difference between T cells derived from normal and tolerance-broken animals was correlated with structural features of the TCR, we undertook an analysis of their Vα and Vβ chains. Since previous stud-

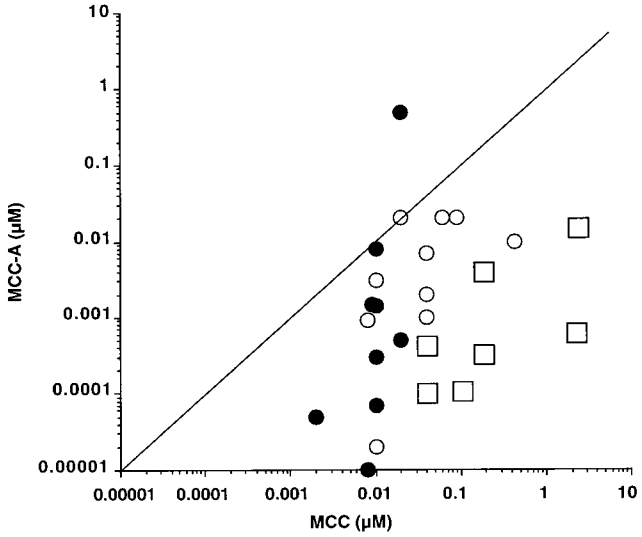

Figure 3. Responsiveness of T cell hybridomas to pMCC and pMCC-A stimulation. T cell hybridomas derived from mice that were pMCC (●) or pMCC-A (○) immunized or mice in which tolerance was terminated by pMCC-A (□) were analyzed with respect to the concentration of pMCC and pMCC-A required to stimulate an IL-2 response to pMCC or pMCC-A. The antigen concentration required for the production of 20 U IL-2 is shown. Each symbol represents an individual T cell hybridoma.

ies of the Vα and Vβ utilization of MCC-specific T cells indicated that the response to this antigen was dominated by T cells that express Vα11 and Vβ3 (32–38), initially flow cytometric analysis of the T cell hybridomas for expression of Vα11 and Vβ3 was performed. Almost one half (5 of 12) of the T cell clones derived from pMCC-immunized animals were $V\alpha11^+/V\beta3^+$, with the rest being either $V\alpha11^+/V\beta3^-$ (4 of 12) or negative for both Vα11 and Vβ3 (3 of 12). The repertoire of clones derived from pMCC-A immune animals differed somewhat from this distribution, in that only about one fourth of the clones (3 of 13) possessed the canonical $V\alpha11^+/V\beta3^+$ phenotype, and about one half (7 of 13) were $V\alpha11^+/V\beta3^-$. The data obtained with the T cell clones derived from the tolerance-broken group were strikingly different, in that all eight clones analyzed were $V\alpha11^+/V\beta3^-$. These data suggest that the typical $V\alpha11^+/V\beta3^+$ MCC-reactive clones were irreversibly inactivated by the tolerizing dose of pMCC and did not participate in the termination of tolerance induced by pMCC-A. Rather, a subset of the $V\alpha11^+/V\beta3^-$ clones responsive to pMCC-A was involved in the breaking of tolerance.

The difference in the repertoire elicited by pMCC and pMCC-A immunization with respect to the relative numbers of $V\beta3^+/V\alpha11^+$ expressing T cells was substantiated by the analysis of short-term T cell lines (Fig. 4). Whereas the T cell line generated from pMCC-immunized animals contained 33% $V\alpha11^+/V\beta3^+$ cells and 25% $V\alpha11^+/V\beta3^-$ cells, the pMCC-A immune line contained less than half the percentage of $V\alpha11^+/V\beta3^+$ cells (14%) and more than twice the percentage of $V\alpha11^+/V\beta3^-$ cells (59%). These data are consistent with the data obtained with the T cell hybridomas derived from pMCC-A–immunized animals that also demonstrated a skewing toward a $V\alpha11^+/V\beta3^-$ phenotype. A second independently derived set of T cell lines gave similar results.

To further analyze the TCR repertoire involved in breaking tolerance and to compare it with the repertoire normally involved in the response to MCC, cDNA sequence analysis of the junctional regions encompassing the CDR3 of the α and β chains was performed, together with the identification of the Vβ families other than Vβ3 that were used (Fig. 5). Previous sequence analyses of the Vα11 genes associated with a cytochrome c–specific response indicated that the CDR3 regions were usually 8 residues long, with a conserved glutamic acid (E) at position 93 and S, A, or G at position 95 (34–38). Of the 8 Vα11 CDR3 regions analyzed from T cell hybridomas derived from pMCC-immunized animals, all had E at position 93, 5 had S at position 95, and 6 were 8 residues in length (Fig. 5 A). Thus, this set of pMCC-specific clones was similar in the structure of their Vα CDR3 regions to previously studied TCRs. The Vα11 CDR3 region from the tolerance-broken group was, in general, very similar to that of the pMCC immune group, but with a slightly greater deviation from the canonical structure (Fig. 5 B). Thus, all 8 clones had E at position 93, but only 4 had G or A at position 95, and only 4 were 8 residues in length. Of particular interest was the finding of a 4-asparagine (N) repeat from positions 95–98 that was present in 4 of the 8 sequences analyzed. This feature was observed in only one of the sequences from the pMCC immune group. The Vα11 CDR3 region from the pMCC-A immune group had characteristics intermediate between the pMCC immune group and the tolerance-broken group (Fig. 5 C): all 10 sequences contained E at position 93, 6 of 10 contained G, A, or S at 95, 6 of 10 had CDR3 regions 8 residues in length, and 3 sequences contained the asparagine repeat from residues 95–98.

With respect to the Vβ gene analysis, as described above, the most striking difference between the pMCC immune group and the tolerance-broken group was the complete absence of Vβ3 in the latter group of hybridomas. 4 of 8 $V\alpha11^+$ TCR genes sequenced in the pMCC immune group were Vβ3, with the other 4 β chains being derived from Vβ8, 14, 15, and 16 gene families (Fig. 5 A). In contrast, the tolerance-broken group (Fig. 5 B) expressed chains from only 2 gene families: Vβ16 (3 of 8) and Vβ8 (5 of 8). The motif and length of the Vβ CDR3 that has been previously described to be associated with anti-cytochrome c activity is a length of 9 residues, with N at position 100 and a G or A at position 102 (34–38). In the pMCC immune group, 6 of 8 β chains analyzed had CDR3 regions 9 residues in length, 6 had N at position 100, and 7 had G or A at 102. In striking contrast to this, none of the 8 Vβ CDR3 regions analyzed from the tolerance-broken group had a length of 9 residues, and none had N at position 100, although 4 of 8 had a G or A at 102. Again, the pMCC-A

A pMCC immunized mice

Vα	CDR3	Jα	a.a	Vβ	CDR3	Jβ	a.a
Vα11	C A A E T S S G Q K L TGTGCTGCT GAGACTTCAAGTGGCCAGAAGCTG	16	8	Vβ3	C A S S P N R G Q D T Q TGTGCCAGC AGCCCAAACAGGGGGCAAGACACCCAG	2.5	9
Vα11	C A A E T S S G Q K L TGTGCTGCT GAGACTTCAAGTGGCCAGAAGCTG	16	8	Vβ3	C A S S P N R G Q D T Q TGTGCCAGC AGCCCAAACAGGGGGCAAGACACCCAG	2.5	9
Vα11	C A A E S S G S W Q L TGTGCTGCT GAGTCTTCTGGCAGCTGTCAACTC	22	8	Vβ3	C A S S L N R G Q D T Q TGTGCCAGC AGTCTTAACAGGGGGCTAGACACCCAG	2.5	9
Vα11	C A A E A H I L L L T A S TGTGCTGCT GAGGCTCACATCCTCCTCCTTACAGCAAGC	50	10	Vβ3	C A S S P N W G Q D T Q TGTGCCAGC AGTCCAAACTGGGGGCAAGACACCCAG	2.5	9
Vα11	C A A E R N N N N A P TGTGCTGCT GAGCGCAATAACAACAATGCCCCA	43	8	Vβ8.3	C A S S D Y G Q G P G V N T L TGTGCCAGC AGTGATTACGGACAGGGGCCGGGGGTGAATACGCTC	1.3	12
Vα11	C A A E A S H Y N V L TGTGCTGCT GAGGCGTCTCATTACAACGTGCTT	21	8	Vβ14	C A W S L N W G Q D T Q TGTGCCTGG AGTCTAAACTGGGGGCAAGACACCCAG	2.5	9
Vα11	C A A E L E H G L P E L TGTGCTGCT GAGCTCGAACACGGGTATCCAGAACTT	49	9	Vβ15	C G V R D D Y A E Q TGTGGTGTT CGGGACGACTATGCTGAGCAT	2.1	7
Vα11	C A A E R S S G N K L TGTGCTGCT GAGAGGAGCAGTGGCAACAAGCTC	32	8	Vβ16	C A S S L N A G R S D Y TGTGCCAGC AGCTTAAACGCGGGGCGCTCCGACTAC	1.2	9

B pMCC tolerized and pMCC-A immunized mice

Vα	CDR3	Jα	a.a	Vβ	CDR3	Jβ	a.a
Vα11	C A A E P N N N N A P TGTGCTGCT GAGCCCAATAACAACAATGCCCCA	43	8	Vβ8.3	C A S S D F P R D G N T L TGTGCCAGC AGTGATTTTCCCAGGGACGGAAATACGCTC	1.3	10
Vα11	C A A E R N N N N A P TGTGCTGCT GAGCGCAATAACAACAATGCCCCA	43	8	Vβ8.1	C A S S D Y G G G T G C A TGTGCCAGC AGTGATTATGGGGGGGGCACCGGGCTAGCT	2.2	10
Vα11	C A A E A A S S G S W Q L TGTGCTGCT GAGGCAGCATCTTCTGGCAGCTGTCAACTC	22	10	Vβ8.3	C G S S I R L G A Q D T Q TGTGCCAGC AGCATTAGACTGGGGGCCCAAGACACCCAG	2.5	10
Vα11	C A A E R N N N N A P TGTGCTGCT GAGCGCAATAACAACAATGCCCCA	43	8	Vβ8.3	C A S S D T G P Y N S P L TGTGCCAGC AGTGATACGGGGCCATATAATTCGCCCCTC	1.6	10
Vα11	C A A E R N N N N A P TGTGCTGCT GAGCGCAATAACAACAATGCCCCA	43	8	Vβ8.3	C A S S D T G A Y N S P L TGTGCCAGC AGTGATACGGGGGCCTATAATTCGCCCCTC	1.6	10
Vα11	C A A E S G T G G Y K V TGTGCTGCT GAGTCCGGGACTGGAGGCTATAAAGTG	12	9	Vβ16	C A S S S G T T S D Y TGTGCCAGC AGCTCGGGGACAACCTCCGACTAC	1.2	8
Vα11	C A A E S G T G G Y K V TGTGCTGCT GAGTCCGGGACTGGAGGCTATAAAGTG	12	9	Vβ16	C A S S Y G T T S D Y TGTGCCAGC AGCTACGGGACAACCTCCGACTAC	1.2	8
Vα11	C A A E A A S S G S W Q L TGTGCTGCT GAGGCCGCATCTTCTGGCAGCTGTCAACTC	22	10	Vβ16	C A S S P D R G G Q D T Q TGTGCCAGC AGCCCCGACAGGGGAGGGCAAGACACCCAG	2.5	10

C pMCC-A immunized mice

Vα	CDR3	Jα	a.a	Vβ	CDR3	Jβ	a.a
Vα11	C A A E A A T G G Y K V TGTGCTGCT GAGGCCGCGACTGGAGGCTATAAAGTG	12	9	Vβ3	C A S S R E Q C N S D Y TGTGCCAGC AGTCGTGAACAATGCAACTCCGACTAC	1.2	9
Vα11	C A A E A A S S G S W Q L TGTGCTGCT GAGGCTGCATCTTCTGGCAGCTGTCAACTC	22	10	Vβ3	C A S S R N W G D D T Q TGTGCCAGC AGTCGTAACTGGGGGGACGACACCCAG	2.5	9
Vα11	C A A E A A T G G Y K V TGTGCTGCT GAGGCCGCGACTGGAGGCTATAAAGTG	12	8	Vβ3	C G S S L N W G D D T Q TGTGCCAGC AGTCTAAACTGGGGGGACGACACCCAG	2.5	9
Vα11	C A A E A N N N N A P TGTGCTGCT GAGGCCAATAACAACAATGCCCCA	43	8	Vβ8.3	C A S S D F G A S A G T L TGTGCCAGC AGTGATTTTGGGGCTAGTGCAGGAACGCTG	2.3	10
Vα11	C A A E R N N N N A P TGTGCTGCT GAGCGCAATAACAACAATGCCCCA	43	8	Vβ8.3	C A S S D W G H N N Q A P TGTGCCAGC AGTGATTGGGGACATAACAACCAGGGTCCG	1.5	10
Vα11	C A A E A S N T N K V TGTGCTGCT GAGGCTTCCAATACCAACAAAGTC	34	8	Vβ16	C A S S Y R E Q G P S D Y TGTGCCAGC AGCTACCGGGAGCAGGGACCCTCCGACTAC	1.2	10
Vα11	C A A E G S S N T N K V TGTGCTGCT GAGGGATCCTCCAATACCAACAAAGTC	34	9	Vβ16	C A S S P G T G A S D Y TGTGCCAGC AGCCCCGGGACAGGGGCCTCCGACTAC	1.2	9
Vα11	C A A E A A T G G Y K V TGTGCTGCT GAGGCCGCGACTGGAGGCTATAAAGTG	12	9	Vβ16	C A S S P G L G G A S E T L TGTGCCAGC AGCCCGGGACTGGGGGCTAGTGCAGAAACGCTG	2.3	11
Vα11	C A A E V N N N N A P TGTGCTGCT GAGGTAAATAACAACAATGCCCCA	43	8	Vβ16	C A S S P G L G G V V Q K R V TGTGCCAGC AGCCCGGGACTGGGGGGGGTAGTGCAGAAACGCGTG	2.3	12
Vα11	C A A E L H N T N K V TGTGCTGCT GAGCGCAATAACAACAATGCCCCA	34	8	Vβ16	C A S S H R D R G I L D Y TGTGCCAGC AGCCACAGGGACAGGGGAATCCTCGATTAC	1.2	10

Figure 4 (continues on facing page).

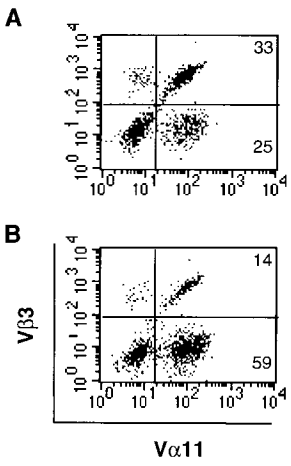

Figure 5. Vα11 and Vβ3 expression in short-term lines from pMCC and pMCC-A immune mice. Short-term T cell lines from a pMCC-immunized animal (A) and pMCC-A–immunized animal (B) were analyzed by flow cytometry for the incidence of $V\alpha11^+$ and/or $V\beta3^+$ cells.

immune group had characteristics intermediate between those of the pMCC immune and tolerance-broken groups. 3 of the 10 β chains analyzed were Vβ3, with the remainder being either Vβ8 (2) or Vβ16 (5). 4 of the 10 CDR3 regions were 9 residues long, 2 had N at position 100, and 6 contained G or A at 102.

These data suggest the following conclusions: (a) MCC recognition, even the low-avidity response associated with the tolerance-broken group, is very strongly associated with the presence of an α chain from the Vα11 family, with a CDR3 region that has an E at position 93, a residue previously implicated as being involved in the interaction with one of the immunodominant TCR contact residues in pMCC, K99 (39); (b) although the expression of Vβ3 is not necessary for MCC reactivity, it appears to be associated with the higher avidity interactions of the pMCC-immunized group; and (c) the lower avidity TCRs found in the tolerance-broken group expressed Vβ8 or Vβ16 and did not contain the canonical N at position 100. Vβ16 expression appeared to be particularly associated with decreased avidity for MCC. Two of the three clones from the pMCC-A immune group that failed to cross-react at all with pMCC were Vβ16, as was the clone with the least cross-reactivity with pMCC. Also, in the tolerance-broken group, the two lowest avidity clones for pMCC (requiring >1 μM MCC for stimulation) expressed Vβ16.

The data shown in Figs. 3 and 5 were obtained with hybridomas derived, in the case of the tolerance-broken group, from a pool of lymphocytes from two animals, and in the case of the normal immune group, from two separate fusions of cells from individual mice. A second fusion of cells from another two tolerance-broken animals was consistent with the data shown in Figs. 3 and 5, in that they had lower avidity for pMCC than normal immune animals, and none of them expressed Vβ3 (data not shown).

Failure of pMCC-A to Reverse Tolerance of Adoptively Transferred pMCC-specific Transgenic T Cells. To evaluate the possibility that breaking tolerance by pMCC-A might involve reversal of anergy, an adoptive transfer system was established in which the effect of pMCC-A on the tolerance induced in a single clone of cells could be evaluated (21). For this purpose, T cells from cytochrome c–specific TCR transgenic mice (AND) were used. The AND TCR is $V\beta3^+/V\alpha11^+$ and recognizes pMCC-A as a heteroclitic antigen. As recipient mice, $H\text{-}2K^+$ invariant chain–deficient animals were used. These animals had previously been shown to lack a T cell repertoire capable of responding to pMCC (40, 41). Thus, if tolerance could be broken in adoptively transferred invariant chain–deficient animals, the T cells involved would have to have been derived from the adoptively transferred TCR transgenic cells. In preliminary experiments, the failure of invariant chain–deficient mice to respond to either pMCC or pMCC-A was confirmed (data not shown). These mice were injected with 2.5×10^6 $CD4^+$ $V\beta3^+/V\alpha11^+$ T cells from AND transgenic mice. 3 d after transfer, one group of mice was tolerized by the intravenous injection of 300 μg of pMCC. 10 d after the induction of tolerance, these animals and groups of nontolerized, adoptively transferred animals were immunized with pMCC or pMCC-A and their immune responses were evaluated by analyzing the in vitro response to pMCC of regional lymph nodes harvested 10 d after immunization. Adoptively transferred and tolerized mice contained $1.61 \pm 0.4\%$ $V\beta3^+/V\alpha11^+$ T cells, compared with $0.72 \pm 0.23\%$ $V\beta3^+/V\alpha11^+$ cells in mice that did not receive an adoptive transfer. This difference was significant ($P < 0.01$), and therefore it could be concluded that after adoptive transfer and tolerance induction, there was a residual transgenic T cell population derived from the adoptively transferred transgenic T cells ($1.61 - 0.72 = 0.89\%$) that could be studied for reversal of anergy. The immune response of T cells derived from adoptively transferred, tolerized, and subsequently immunized animals is shown in Fig. 6. The data, expressed as the amount of IFN-γ per $V\beta3^+/V\alpha11^+$ cell put into culture, indicated: (a) tolerance could be established in this adoptive transfer system, since animals that received an intravenous injection of pMCC before immunization with pMCC in CFA were severely impaired in their capacity to produce IFN-γ compared with the nontolerized controls (0.15 vs. 0.95 pg/cell); and (b) immunization with the heteroclitic analogue pMCC-A failed to break this tolerant state.

TCR Antagonist Peptides Can Act as Agonists When Used to Stimulate Recently Primed T Cells. If the model suggested by our data is correct, namely that breaking tolerance involves the activation of T cells with low affinity for the tolerogen but high affinity for the heteroclitic antigen, then it must be further postulated that these T cells, once stimu-

Figure 4 (continued). Analysis of cytochrome c–specific TCRs. pMCC-reactive $V\alpha11^+$ T cell hybridomas derived from (A) pMCC-immunized mice, (B) pMCC-tolerized and pMCC-A–immunized mice, and (C) pMCC-A immunized mice were analyzed for their Vα and Vβ CDR3 sequence, J region usage, length of CDR3 region, and Vβ gene family usage.

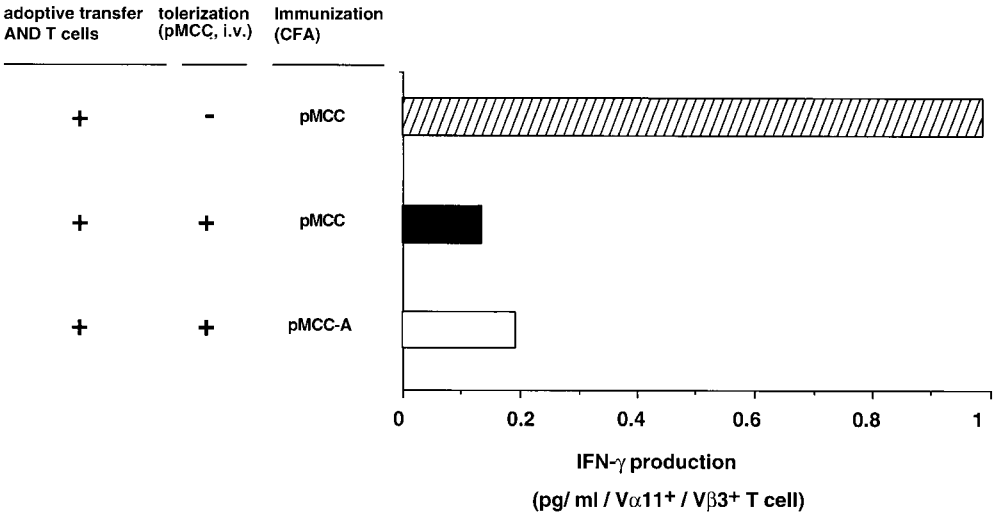

Figure 6. Failure to break tolerance of adoptively transferred pMCC-specific transgenic T cells. Lymph node cells from AND transgenic T cells were injected into invariant chain–deficient B10.BR mice. Animals were left untreated or were tolerized by the intravenous injection of 300 μg pMCC. Mice were subsequently immunized with pMCC or pMCC-A and analyzed for their responsiveness to pMCC by measuring their capacity to produce IFN-γ upon restimulation in vitro. Data are plotted as the amount of IFN-γ produced per $V\alpha11^+/V\beta3^+$ cell placed into culture.

lated by the heteroclitic antigen, have a lowered threshold for stimulation and become responsive to stimulation by the lower affinity tolerogen. To evaluate this postulate, we studied the response of naive and recently primed TCR transgenic T cells to stimulation with either the cognate antigen used to prime the T cells or a nonantigenic TCR antagonist analogue of the antigen with presumably lower affinity for the TCR. T cells from the cytochrome c–specific TCR transgenic line AD10 were used for this purpose, since this TCR had been extensively analyzed previously for reactivity to a large panel of pMCC analogue peptides. Naive T cells or T cells that had been stimulated with pMCC 8–12 d previously were stimulated with varying doses of pMCC or the TCR antagonist peptide T102G, which has a glycine (G) substituted for one of the major TCR contact residues, T102. The data shown in Fig. 7 demonstrate that the T102G analogue, which was nonantigenic for naive T cells, was an agonist when assayed

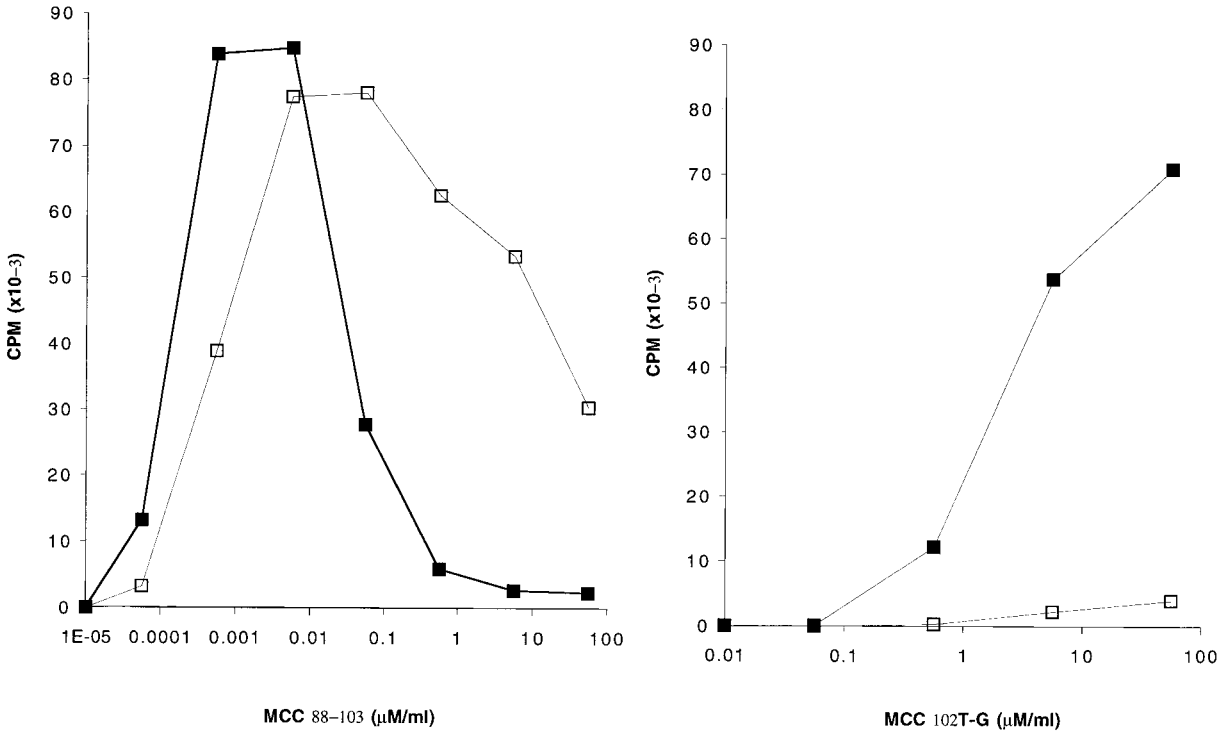

Figure 7. Capacity of previously primed T cells to respond to TCR antagonist peptides. Naive AD10 transgenic T cells (□) or AD10 cells that had been stimulated with 0.1 μg/ml of pMCC 12 d previously (■) were stimulated with various quantities of pMCC (left) or the TCR antagonist peptide, T102G (right), in the presence of CH27 cells as APCs. Proliferation was measured by the incorporation of [^{3}H]thymidine added at day 2 of a 3-d culture.

on recently primed T cells, albeit requiring a high concentration (1–100 μg/ml) to achieve this effect.

Discussion

This study was undertaken to evaluate two potential mechanisms by which antigen analogues might operate in the termination of T cell tolerance to an immunodominant epitope: stimulation of low-affinity nontolerized clones and reversal of anergy. Three sets of data presented in this study favor the conclusion that the first mechanism is operative.

First, T cell hybridomas derived from tolerance-broken animals required, on average, 88-fold higher concentration of antigen than hybridomas derived from normal immune animals in order to be stimulated to produce IL-2. This difference in antigen dose requirement is most likely due to differences in affinity of the TCRs from these two groups of hybridomas, but other possibilities need to be considered, such as differences in the levels of expression of molecules that contribute to the stimulation of the T cells; these include the TCR itself, the CD4 coreceptor, and adhesion molecules such as LFA-1. Two lines of evidence suggest that differential expression of these molecules does not contribute to the antigen dose requirements observed. First, no correlation between the levels of expression of TCR and CD4 and the antigen dose requirement for stimulation was observed (Fig. 2; and data not shown). Second, when I-E^k–transfected fibroblasts that lacked intercellular adhesion molecule (ICAM), B-7, or other known ligands for T cell adhesion/costimulator receptors were used as APC, the large difference in antigen dose requirements for the tolerance-broken and normal immune animals was still observed (576 vs. 22 nM).

The second set of data that supports the hypothesis of stimulation of low-affinity nontolerized clones as a mechanism for breaking tolerance is the finding of a different repertoire of TCRs on T cells from tolerance-broken animals and normal immune animals. Although there was no absolutely unique feature to the TCRs from the tolerance-broken group of hybridomas, there was pronounced skewing of the TCRs compared with those found in the normal immune group. Vβ3, the major Vβ family found in the normal immune group (33–38), was not represented at all in the tolerance-broken group. Instead, Vβ8 and Vβ16 were expressed in the tolerance-broken group—Vβ families that were only occasionally expressed in the MCC-specific T cells from normal immune animals. Similarly, the canonical Vβ CDR3 motif and length associated with the response to pMCC were not observed in any of the Vβ CDR3 regions from the tolerance-broken group of hybridomas. If reversal of anergy were the mechanism for breaking tolerance, there is no apparent reason for it to be restricted to a minor subset of clones with the exclusion of the major $V\beta3^+/V\alpha11^+$ subset. On the other hand, a distinctly different repertoire of TCRs with different Vβ/Vα composition would be consistent with a mechanism of breaking tolerance that involved the stimulation of low-affinity clones that were not capable of being stimulated after MCC immunization nor were tolerizable when MCC was administered in a tolerogenic form.

The third observation in support of the concept that tolerance was broken by low-affinity nontolerized clones was our failure to terminate the tolerant state of adoptively transferred and tolerized transgenic T cells. When transgenic T cells bearing a typical $V\beta3^+/V\alpha11^+$ TCR specific for MCC were transferred and subsequently tolerized to MCC, the same heteroclitic peptide that was capable of breaking tolerance in conventional animals failed to break tolerance in these animals with a clonal population of tolerized cells.

Taken together, these three sets of data strongly support the hypothesis that breaking tolerance involves the stimulation of nontolerized, low-affinity clones by the MCC heteroclitic analogue. For reversal of anergy to be operative, it would be necessary to postulate that certain ($V\beta3^+/V\alpha11^+$) cells are selectively incapable of undergoing reversal of anergy, whereas others ($V\beta8^+/V\alpha11^+$ and $V\beta16^+/V\alpha11^+$) are able to undergo anergy reversal. Although this is theoretically possible, we consider it unlikely.

Our data are consistent with previous reports that found that tolerance to certain self-epitopes was incomplete and that immunization with the cognate antigen could generate an immune response (42–44). However, the responding T cells that were elicited were of relatively low affinity. For instance, immunization of beef insulin (BI) transgenic mice with BI generated a BI-specific T cell response that required higher concentrations of antigen to be elicited than the response elicited in nontransgenic mice (42). Similarly, low-avidity CD8 T cell responses were obtained after immunization with a p53 self-epitope when $p53^+$ mice were used. In contrast, p53-deficient mice generated a higher avidity response (43). These reports suggest that when self-tolerance is incomplete, active immunization with the unaltered self-protein or an epitope derived from it can elicit a response of low-avidity T cells that had escaped tolerance. Our data extend these observations to situations in which tolerance to the epitope in question is complete and cannot be reversed by immunization with the native epitope but can be overcome by immunization with a heteroclitic analogue.

The hypothesis that termination of tolerance to pMCC involves the stimulation of low-affinity clones that are neither tolerized nor stimulated by pMCC but can be stimulated by the heteroclitic analogue, pMCC-A, requires the further postulate that after stimulation by the heteroclitic antigen, the affinity threshold for stimulation is lowered such that restimulation with the tolerogen results in a recall response. To test this postulate, we studied the response of naive and previously primed MCC-specific transgenic T cells to a ligand of presumably lower affinity, a TCR antagonist peptide. It was found that, whereas naive and primed T cells responded with similar vigor to the wild-type agonist peptide, only primed cells proliferated in response to stimulation with the antagonist peptide. This finding further supports the low-affinity T cell model of breaking tolerance. The mechanism by which priming results in a low-

ering of the affinity threshold for TCR-mediated stimulation is unknown. One possible factor that could influence the sensitivity of the T cell response is the level of expression of the CD4 coreceptor. Several studies have documented that T cell clones with the same TCR have heightened responses to suboptimal concentrations of agonist peptide when the T cell is $CD4^+$ compared with $CD4^-$ variants or when $CD4^+$ cells are pretreated with anti-CD4 antibodies (45–48). Although we have not been able to document any differences in the level of expression of CD4 on the naive and recently primed T cells that we analyzed (data not shown), it is possible that CD4 function differs in the two cell types; e.g., the activity of the CD4-associated Lck may be greater in primed compared with naive T cells. This and other possible differences between naive and recently primed T cells that may be important in the conversion of TCR antagonists into agonists are being investigated.

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References

1. Oldstone, M.B.A. 1990. Molecular mimicry and autoimmune disease. *Cell*. 50:819–820.
2. Luo, A.-M., K.M. Garza, D. Hunt, and K.S.K. Tung. 1993. Antigen mimicry in autoimmune disease. Sharing of amino acid residues critical for pathogenic T cell activation. *J. Clin. Invest*. 92:2117–2123.
3. Wucherpfennig, K.W., and J.L. Strominger. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell*. 80:695–705.
4. Loftus, D.J., C. Castelli, T.M. Clay, P. Squarcina, F.M. Marincola, M.I. Nishimura, G. Parmiani, E. Appella, and L. Rivoltini. 1996. Identification of epitope mimics recognized by CTL reactive to the melanoma/melanocyte–derived peptide $MART\text{-}1_{(27–35)}$. *J. Exp. Med*. 184:647–657.
5. Hemmer, B., B.T. Fleckenstein, M. Vergelli, G. Jung, H. McFarland, R. Martin, and K.-H. Wiesmuller. 1997. Identification of high potency microbial and self ligands for a human autoreactive class II–restricted T cell clone. *J. Exp. Med*. 185:1651–1659.
6. Barnaba, V., and F. Sinigaglia. 1997. Molecular mimicry and T cell–mediated autoimmune disease. *J. Exp. Med*. 185: 1529–1531.
7. Zhao, Z.-S., F. Granucci, L. Yeh, P.A. Schaffer, and H. Cantor. 1998. Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. *Science*. 279: 1344–1347.
8. Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell*. 49:273–280.
9. Kisielow, P., H. Bluthmann, U.D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature $CD4^+8^+$ thymocytes. *Nature*. 333:742–746.
10. Sha, W.C., C.A. Nelson, R.D. Newberry, D.M. Kranz, J.H. Russell, and D.Y. Loh. 1988. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature*. 336:73–76.
11. Bloom, B.R., R.L. Modlin, and P. Salgame. 1992. Stigma variations: observations on suppressor T cells and leprosy. *Annu. Rev. Immunol*. 10:453–488.
12. Karpus, W.J., J.D. Peterson, and S.D. Miller. 1994. Anergy in vivo: down-regulation of antigen-specific $CD4^+$ Th1 but not Th2 cytokine responses. *Int. Immunol*. 6:721–730.
13. Nicholson, L.B., J.M. Greer, R.A. Sobel, M.B. Lees, and V.J. Kuchroo. 1995. An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. *Immunity*. 3:397–405.
14. Saoudi, A., S. Simmond, I. Huitinga, and D. Mason. 1995. Prevention of experimental allergic encephalomyelitis in rats by targeting autoantigen to B cells: evidence that the protective mechanism depends on changes in the cytokine response and migratory properties of the autoantigen-specific T cells. *J. Exp. Med*. 182:335–344.
15. Forsthuber, T., H.C. Yip, and P.V. Lehmann. 1996. Induction of TH1 and TH2 immunity in neonatal mice. *Science*. 271:1728–1730.
16. Gammon, G., and E. Sercarz. 1989. How some T cells escape tolerance induction. *Nature*. 342:183–185.
17. Benichou, G., P.A. Takizawa, P.T. Ho, C.C. Killion, C.A. Olson, M. McMillan, and E.E. Sercarz. 1990. Immunogenicity and tolerogenicity of self-major histocompatibility complex peptides. *J. Exp. Med*. 172:1341–1346.

18. Agrawal, B., M. Manickasundari, E. Fraga, and B. Singh. 1991. T cells that recognize peptide sequences of self MHC class II molecules exist in syngeneic mice. *J. Immunol*. 147: 383–390.
19. Cibotti, R., J.M. Kanellopoulos, J.P. Cabaniols, O. Halle-Panenko, K. Kosmatopoulos, E. Sercarz, and P. Kourilsky. 1992. Tolerance to a self-protein involves its immunodominant but does not involve its subdominant determinants. *Proc. Natl. Acad. Sci. USA*. 89:416–420.
20. Mamula, J. 1993. The inability to process a self-peptide allows autoreactive T cells to escape tolerance. *J. Exp. Med*. 177:567–571.
21. Kearney, E.R., K.A. Pape, D.Y. Loh, and M.K. Jenkins. 1994. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity*. 1:327–339.
22. Van Houten, N., and S.F. Blake. 1996. Direct measurement of anergy of antigen-specific T cells following oral tolerance induction. *J. Immunol*. 157:1337–1341.
23. Falb, D., T.J. Briner, G.H. Sunshine, C.R. Bourque, M. Luqman, M.L. Gefter, and T. Kamradt. 1996. Peripheral tolerance in T cell receptor-transgenic mice: evidence for T cell anergy. *Eur. J. Immunol*. 26:130–135.
24. Lanoue, A., C. Bona, H. von Boehmer, and A. Sarukhan. 1997. Conditions that induce tolerance in mature $CD4^+$ T cells. *J. Exp. Med*. 185:405–414.
25. Pape, K.A., R. Merica, A. Mondino, A. Khoruts, and M.K. Jenkins. 1998. Direct evidence that functionally impaired $CD4^+$ T cells persist in vivo following induction of peripheral tolerance. *J. Immunol*. 160:4719–4729.
26. Zügel, U., R. Wang, G. Shih, A. Sette, J. Alexander, and H.M. Grey. 1998. Termination of peripheral tolerance to a T cell epitope by heteroclitic antigen analogues. *J. Immunol*. 161:1705–1709.
27. La Face, D.M., C. Couture, K. Anderson, G. Shih, J. Alexander, A. Sette, T. Mustelin, A. Altman, and H.M. Grey. 1997. Differential T cell signaling induced by antagonist peptide-MHC complexes and the associated phenotypic responses. *J. Immunol*. 158:2057–2064.
28. Sette, A., S. Southwood, D. O'Sullivan, F.C.A. Gaeta, J. Sidney, and H.M. Grey. 1992. Effect of pH on class II-peptide interactions. *J. Immunol*. 148:844–851.
29. Ignatowicz, L., J. Kappler, and P. Marrack. 1996. The repertoire of T cells shaped by a single MHC/peptide ligand. *Cell*. 84:521–529.
30. Candélas, S., J. Katz, C. Benoist, D. Mathis, and K. Haskins. 1991. Islet-specific T-cell clones from nonobese diabetic mice express heterogeneous T-cell receptors. *Proc. Natl. Acad. Sci. USA*. 88:6167–6170.
31. Pannetier, C., M. Cochet, S. Darche, A. Casrouge, M. Zöller, and P. Kourilsky. 1993. The sizes of the CDR3 hypervariable regions of the murine T-cell receptor β chains vary as a function of the recombined germ-line segments. *Proc. Natl. Acad. Sci. USA*. 90:4319–4323.
32. Sorger, S.B., Y. Paterson, P.J. Fink, and S.M. Hedrick. 1990. TCR junctional regions and the MHC molecule affect the recognition of antigenic peptides by T cell clones. *J. Immunol*. 144:1127–1135.
33. Jorgensen, J.L., P.A. Reay, E.W. Ehrich, and M.M. Davis. 1992. Molecular components of T-cell recognition. *Annu. Rev. Immunol*. 10:835–873.
34. Nakano, N., R. Rooke, C. Benoist, and D. Mathis. 1997. Positive selection of T cells induced by viral delivery of neopeptides to the thymus. *Science*. 275:678–683.
35. Kaye, J., G. Kersh, I. Engel, and S.M. Hedrick. 1991. Structure and specificity of the T cell antigen receptor. *Semin. Immunol*. 3:269–281.
36. Hedrick, S.M., I. Engel, D.L. McElligott, P.J. Fink, M.L. Hsu, D. Hansburg, and L.A. Matis. 1988. Selection of amino acid sequences in the beta chain of the T cell antigen receptor. *Science*. 239:1541–1544.
37. Cochet, M., C. Pannetier, A. Regnault, S. Darche, C. Leclerc, and P. Kourilsky. 1992. Molecular detection and in vivo analysis of the specific T cell response to a protein antigen. *Eur. J. Immunol*. 22:2639–2647.
38. McHeyzer-Williams, M.G., and M.M. Davis. 1995. Antigen-specific development of primary and memory T cells in vivo. *Science*. 268:106–111.
39. Jorgensen, J.L., U. Esser, B. Fazekas de St. Groth, P.A. Reay, and M.M. Davis. 1992. Mapping T-cell receptor-peptide contacts by variant peptide immunization of single-chain transgenics. *Nature*. 355:224–230.
40. Viville, S., J. Neefjes, V. Lotteau, A. Dierich, M. Lemeur, H. Ploegh, C. Benoist, and D. Mathis. 1993. Mice lacking the MHC class-II associated invariant chain. *Cell*. 72:635–648.
41. Tourne, S., N. Nakano, S. Viville, C. Benoist, and D. Mathis. 1995. The influence of invariant chain on the positive selection of single T cell receptor specificities. *Eur. J. Immunol*. 25:1851–1856.
42. Poplonski, L., B. Vukusic, J. Pawling, S. Clapoff, J. Roder, N. Hozumi, and J. Wither. 1996. Tolerance is overcome in beef insulin-transgenic mice by activation of low-affinity autoreactive T cells. *Eur. J. Immunol*. 26:601–609.
43. Theobald, M., J. Biggs, J. Hernández, J. Lustgarten, C. Labadie, and L.A. Sherman. 1997. Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. *J. Exp. Med*. 185:833–841.
44. Targoni, O.S., and P.V. Lehmann. 1998. Endogenous myelin basic protein inactivates the high avidity T cell repertoire. *J. Exp. Med*. 187:2055–2063.
45. Marrack, P., R. Endres, R. Shimonkevitz, A. Zlotnik, D. Dialynas, F.W. Fitch, and J. Kappler. 1983. The major histocompatibility complex–restricted antigen receptor on T cells. II. Role of the L3T4 product. *J. Exp. Med*. 158:1077–1091.
46. Mannie, M.D., J.M. Rosser, and G.A. White. 1995. Autologous rate myelin basic protein is a partial agonist that is converted into a full antagonist upon blockade of CD4. *J. Immunol*. 154:2642–2654.
47. Hampl, J., Y. Chien, and M.M. Davis. 1997. CD4 augments the response of a T cell to agonist but not to antagonist ligands. *Immunity*. 7:379–385.
48. Vidal, K., B.L. Hsu, C.B. Williams, and P.M. Allen. 1996. Endogenous altered peptide ligands can affect peripheral T cell responses. *J. Exp. Med*. 183:1311–1321.