ANALYSIS OF Mls^c GENETICS

A Novel Instance of Genetic Redundancy

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The mature T cell repertoire is thought to be shaped by two events that occur during maturation of the immune system: negative selection, which functions to eliminate high affinity self-reactive immune-competent cells, and positive selection, which results in selection of T cells with affinity for foreign antigens in association with self MHC gene products (1, 2). The identification of the molecules that play a critical role as ligands for the TCR in these events has been an important issue in the further characterization of these processes. Gene products encoded by the MHC have been widely accepted as relevant ligands for both positive and negative selection. In addition to MHC gene products, however, an important role of self non-MHC gene products for T cell repertoire establishment has been proposed (3, 4). Recent observation of peptide-like structures associated with class I molecules by X ray crystallography provided direct evidence consistent with involvement of self non-MHC products in T cell recognition (5). Furthermore, the involvement of non-MHC gene products in T cell recognition of MHC gene products has been suggested by the observation that certain Vβ TCR (Vβ17a [6, 7], Vβ11 [8]) appear to react preferentially to I-E molecules in association with other self determinants.

Although numerous non-MHC gene products are expressed on lymphocytes, minor lymphocytes stimulating (Mls) determinants are unique among them in the extraordinarily high precursor frequency of naive T cells reactive to Mls determinants (9, 10). The Mls system has consequently been extremely informative in studies of self tolerance in the T cell repertoire (11–17). Recently, it was demonstrated that Vβ6 (14) and Vβ8.1 (13) TCR expression are strongly associated with T cell recognition of Mls^a determinants. Moreover, these determinants play the role of ligand in the elimination of self-reactive T cells as evidenced by the deletion of Vβ6- and Vβ8.1-expressing T cells in Mls^a-positive strains. Similarly, expression of Mls^c has recently been shown to be associated with Vβ3 expression (18); and V$\beta3^+$ T cells are efficiently deleted in Mls^c strains (16, 17). Although this accumulated evidence is consistent with an important biological role of Mls products in the immune system, Mls-like determinants have to date been observed only in the mouse, and no equivalent system has as yet been identified in other species such as man.

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[1] *Abbreviations used in this paper:* FCM, flow cytometry analysis; MMC, mitomycin C; TCR, Texas-red streptavidin.

In this work, we describe a unique feature of the genetic control of the expression of Mls^c determinants. Unlike other systems such as MHC or Mls^a, in which a cell surface determinant is encoded by a single locus or by two complementing loci, Mls^c gene products appear to be controlled by at least two unlinked loci, either one of which is sufficient to encode an indistinguishable Mls^c determinant. Determinant(s) encoded by either locus are fully capable of evoking Mls^c-specific T cell responses and of functioning as ligands in the deletion of self-Mls^c-reactive Vβ3$^+$ T cells. This finding of a unique redundancy in the genes encoding Mls^c determinants is discussed in terms of its implications for the nature of Mls determinants and in terms of the potential existence of Mls-like gene products in other species.

Materials and Methods

Mice. AKR/J, B10.BR, CBA/J, and DBA/2 mice were purchased from The Jackson Laboratory, Bar Harbor, ME. (AKR/J × C3H/HeJ)F$_1$, AKR/J × (AKR/J × C3H/HeJ)F$_1$ (AKR/J × C3H/HeJ)F$_1$ × B10.BR, (B10.BR × CBA/J)F$_1$, (B10.BR × CBA/J)F$_1$ × B10.BR, (C3H/HeJ × B10.BR)F$_1$, and (C3H/HeJ × B10.BR)F$_1$ × B10.BR mice were bred in our facilities.

Antibody. KJ25 (16), a hamster mAb specific for the Vβ3 chain of the murine TCR, was kindly provided to us by Drs. Philippa Marrack and John W. Kappler (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). Goat anti-mouse IgD antiserum was kindly provided by Dr. Fred D. Finkelman (Uniformed Services University of the Health Science, Bethesda, MD). The use of this antibody has been described elsewhere (19).

T Cell Clones and T Cell Proliferation Assay. The generation and characterization of Mls^a-, Mls^c-, and I-A^k-reactive clones have been described (20, 21). T cell proliferation was assayed as previously described (20). Results are expressed as the arithmetic means of triplicate cultures and the SEs were generally <10% of the mean. Responses that were greater ($p < 0.025$ based on the Student's t test) than responses to syngeneic stimulators were regarded as positive. To normalize the results obtained from multiple experiments, the T cell responses are described as percent of a control response in each experiment. Details are described in the figure legends.

Flow Cytometry Analysis (FCM)[1]. FCM was performed by using a modified FACS II (Becton Dickinson & Co., Mountain View, CA). Enriched splenic T cells were obtained from spleen cells by passage over rabbit anti-mouse Ig-coated plates. Cells were incubated with saturating amounts of mAb culture supernatant for 30 min at 4°C, washed, and treated with FITC-coupled goat anti-hamster antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Cells were washed again and resuspended for FCM analysis. For two-color analysis, the cells were first incubated with culture supernatant of anti-Vβ mAb, washed, and stained for 30 min at 4°C using FITC-coupled goat anti-hamster antibody. The cells were then washed and incubated with biotin-labeled anti-Thy-1.2 (Becton Dickinson Immunocytometry Systems, Mountain View, CA) and counterstained with Texas red-streptavidin (TR) (Bethesda Research Laboratories, Bethesda, MD). Two-color immunofluorescence data were displayed as contour diagrams in which log intensities of green (FITC) fluorescence were plotted on the x-axis and log intensities of red (TR) fluorescence were plotted on the y-axis. The percentages of Vβ3$^+$ T cells were calculated by dividing the number of Thy-1$^+$ Vβ3$^+$ cells by the total number of all Thy-1$^+$ cells.

Results

Mls^c Expression by Spleen Cells from (AKR/J × C3H/HeJ)F$_1$ × B10.BR Offspring. To investigate the allelism between genes encoding Mls^a and Mls^c, the progeny of an (Mls^a × Mls^c)F$_1$ × Mls^b breeding were phenotyped for expression of Mls^a and Mls^c determinants. The results of a representative experiment are shown in Table I. Irradiated spleen cells from (AKR/J × C3H/HeJ)F$_1$ × B10.BR ([Mls^a × Mls^c])F$_1$ ×

TABLE I

Mls Expression by Spleen Cells from (AKR/J × C3H/HeJ)F1 × B10.BR Mice

Stimulator*	Responder T cells‡ C3H/HeJ (anti-Mls^a)	AKR/J (anti-Mls^c)	Mls type§ a	c
AKR/J	277,306‖	2,585	+	−
C3H/HeJ	10,308	62,242	−	+
(AKR × C3H)F1	356,396	25,061	+	+
(AKR × C3H)F1 × B10.BR				
BC 1	240,295	2,094	+	−
2	11,201	5,455	−	+
3	252,109	17,431	+	+
4	12,023	9,537	−	+
5	11,903	5,286	−	+
6	292,223	10,731	+	+
7	12,611	7,416	−	+
8	152,880	7,601	+	+
9	150,780	5,396	+	+
10	14,400	5,189	−	+
11	12,273	10,225	−	+
12	4,707	8,185	−	+
13	3,806	2,287	−	−
14	143,094	8,016	+	+
15	90,707	5,609	+	+
16	14,397	12,013	−	+

* 5×10^5 mitomycin C-treated stimulator cells were added to each culture.

‡ 3×10^5 unprimed nylon nonadherent spleen cells were cultured with the stimulator cells.

§ Typing of Mls was based on the experimental results shown.

‖ Arithmetic mean of [^{3}H]TdR incorporation of triplicate cultures. Underscored numbers are those that are significantly greater ($p < 0.025$ based on the student's t test) than responses to syngeneic stimulators.

Mls^b) offspring were cocultured with C3H/HeJ T cells (for detection of Mls^a) or with AKR/J T cells (for detection of Mls^c). As previously reported (22), some offspring expressed neither Mls^a nor Mls^c and others expressed both Mls^a and Mls^c, consistent with the conclusion that the genes encoding Mls^a and Mls^c determinants are nonallelic. However, it was also observed that while 7 of 16 progeny expressed Mls^a determinants (~50%, consistent with the control of Mls^a expression by a single gene locus), stimulator cells from 14 of 16 progeny expressed Mls^c. This high frequency of Mls^c-positive offspring suggested that Mls^c determinants as detected by heterogeneous responding T cell populations are encoded by two or more unlinked genes. These results did not distinguish whether the observed Mls^c-specific T cell responses might be the sum of responses of different sets of T cells that are reactive to different "Mls^c determinants" or whether the Mls^c-specific T cell responses are responses to a single Mls^c determinant that may be encoded by any one of two or more unlinked genes. To address these alternative possibilities, a segregation analysis was performed using a panel of Mls^c-specific clones.

Segregation Analysis of Genes Encoding Mls^c Determinants in C3H/HeJ and CBA/J

Mice. First, the response patterns of three different Mls^c-specific T cell clones (BC3B13, BC3C13, and BCAC5) (20, 21) to splenic stimulators derived from an AKR/J × (AKR/J × C3H/HeJ)F$_1$ ([Mls^a × Mls^c] × Mls^a) backcross were examined. The rationale behind this experiment was the following. If Mls^c-specific responses to C3H/HeJ-derived stimulators consist of responses to multiple Mls^c determinants, then it would be expected that any one clone would be reactive to only one Mls^c determinant and that individual clones would respond to some but not other Mls^c-positive offspring of the indicated backcross. In contrast, if Mls^c is a single determinant controlled by multiple gene loci, then all Mls^c-specific clones would respond to all Mls^c-positive offspring.

As shown in Fig. 1, all three Mls^c-specific clones exhibited similar response patterns to stimulator cells from individual AKR/J × (AKR/J × C3H/HeJ)F$_1$ backcross mice. Stimulator cells from two backcross animals (Nos. 1 and 4) were non-

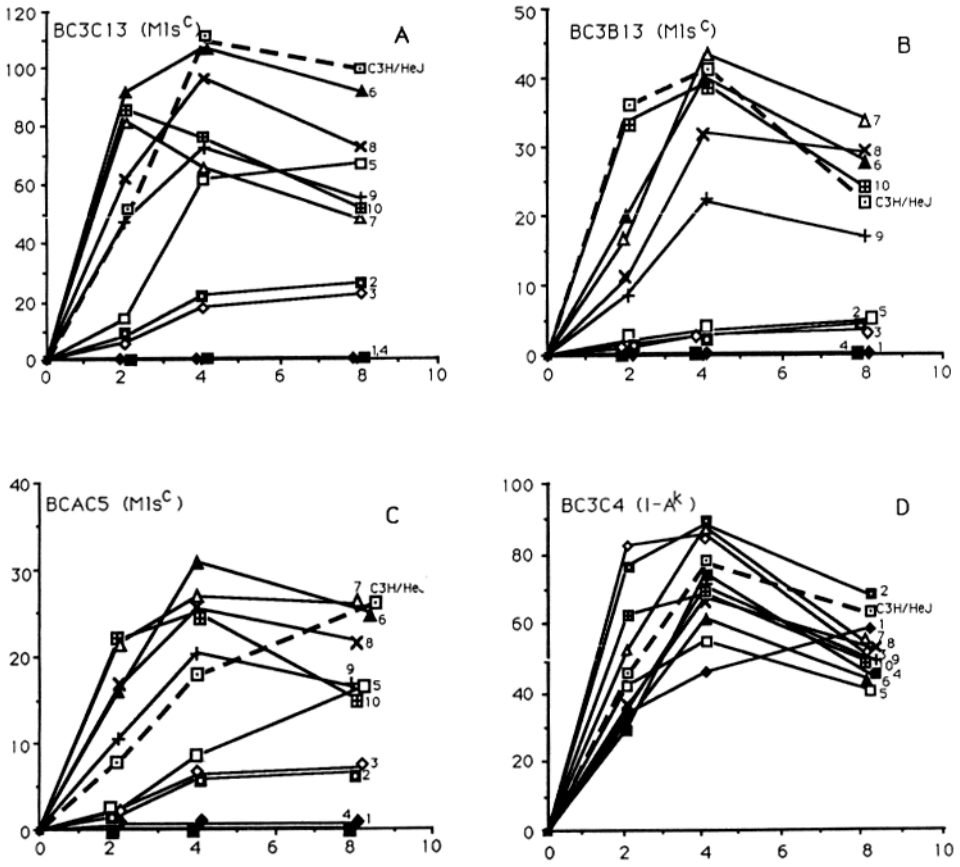

FIGURE 1. Responses of Mls^c-specific clones to AKR/J × (AKR/J × C3H/HeJ)F$_1$ backcross mice. 10^4 cloned T cells were cultured with varying numbers of splenic stimulator cells from individual animals, as well as C3H/HeJ, which were inactivated with mitomycin C (MMC), as described elsewhere (20). Each point represents the mean of [^{3}H] TdR incorporation of triplicate cultures. (*A*) BC3C13, (*B*) BC3B13, and (*C*) BCAC5 are Mls^c specific, and (*D*) BC3C4 is an I-A^k-specific clone.

stimulatory for all three Mls^c-reactive clones over widely titrated numbers of stimulators. Each of the three clones responded to stimulators from the other 8 of 10 backcross mice tested, although two animals (Nos. 2 and 3) revealed consistently weak stimulation. The differences in Mls^c-stimulating capacity of stimulator cells from these backcross animals did not appear to reflect differences in the overall condition of stimulator cells, because all populations were capable of efficiently stimulating an I-A^k-specific T cell clone, BC3C4. These results thus favor a single determinant model of Mls^c recognition.

To further assess the genetic control of Mls^c expression, more extensive segregation analysis was carried out by testing the response pattern of Mls^c-specific clones to splenic stimulator cells derived from the offspring of a number of breeding combinations. To normalize the responses of a clone in multiple experiments, all responses were expressed as the percent of the response of the same clone to C3H/HeJ stimulators in that experiment. As shown in Fig. 2, 18 of 24 AKR/J × (AKR/J × C3H/HeJ)F_1 backcross animals were stimulatory to the Mls^c-specific clone BC3C13, although the strength of Mls^c stimulatory abilities of individual animals varied among these Mls^c-positive stimulators. This number of Mls^c-positive offspring (18/24, 75%) is not consistent with the number expected based on the segregation of a single locus encoding Mls^c in C3H/HeJ ($p < 0.025$).

An additional segregation analysis was performed using (AKR/J × C3H/HeJ)F_1 × B10.BR ([Mls^a × Mls^c]F_1 × Mls^b). This breeding combination allows an analysis of the segregation of both Mls^a-encoding and Mls^c-encoding genes. As shown in Fig. 3, among 70 offspring examined in five experiments, 31 (44.3%) were identified as Mls^a-positive (Fig 3 *A*) based on the responses of the Mls^a-specific clone BARB12 (20), consistent with the control of the Mls^a determinant by a single locus in the AKR/J strain ($p > 0.10$). In contrast, 52 offspring were stimulatory for the Mls^c-

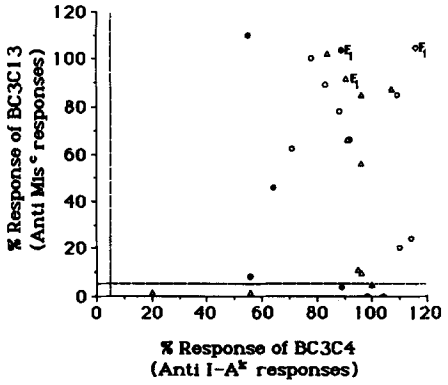

FIGURE 2. Mls^c expression by AKR/J × (AKR/J × C3H/HeJ)F_1 backcross animals. The vertical axis represents the response of Mls^c-specific clone BC3C13 to each backcross offspring expressed as a percentage of the response of that clone to C3H/HeJ stimulators in the same experiment. The horizontal dotted line represents the mean BC3C13 response to syngeneic B10.BR expressed as a percentage of the response to C3H/HeJ in three experiments. On the horizontal axis is indicated the response of the I-A^k-specific clone BC3C4 to each backcross offspring expressed as a percentage of the response of that clone to C3H/HeJ in the same experiment. The vertical dotted line indicates the mean sum of ^3H uptake by BC3C4 alone and stimulator alone expressed as a percentage of the response to C3H/HeJ in three experiments. Each point represents the maximum response of a clone tested for responses to three different doses of each splenic stimulator cell population. Each symbol indicates the results obtained from an individual mouse in the same experiment (○, Exp. 1; △, Exp. 2; and ●, Exp. 3). (Exp. 1 and 3) Splenic stimulator cells were obtained from mice that had been injected with 200 μl anti-mouse IgD antiserum 24 h before mice were killed and cells were inactivated by 3,000-rad irradiation. (Exp. 2) Splenic stimulator cells from untreated animals were treated with MMC. Maximum responses of clones to C3H/HeJ in each experiment were the following: (Exp. 1) BC3C13 = 104,018, BC3C4 = 53,231; (Exp. 2) BC3C13 = 47,240, BC3C4 = 27,567; (Exp. 3) BC3C13 = 131,225, BC3C4 = 39,952.

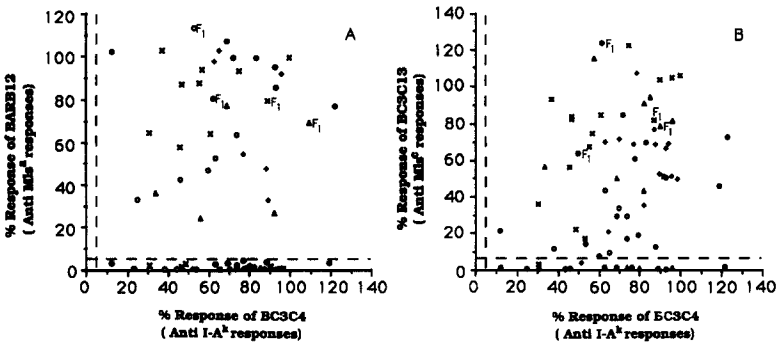

FIGURE 3. Mls expression by (AKR/J × C3H/HeJ)F_1 × B10.BR offspring. The vertical axis represents the response of Mls^a-specific clone BARB12 (*A*) or the response of Mls^c-specific clone BC3C13 (*B*) to each offspring expressed as a percentage of the response of that clone to AKR/J stimulators (*A*) or C3H/HeJ stimulators (*B*), respectively, in the same experiment. The horizontal dotted line represents the mean BARB12 (*A*) or BC3C13 (*B*) response to syngeneic B10.BR expressed as a percentage of the response to AKR/J (*A*) or C3H/HeJ (*B*) in three experiments. On the horizontal axis is indicated the response of the I-A^k-specific clone BC3C4 to each offspring expressed as a percentage of the response of that clone to AKR/J (*A*) or C3H/HeJ (*B*) in the same experiment. The vertical dotted line indicates the mean sum of ^3H uptake by BC3C4 alone and stimulator alone expressed as percentage of the response to AKR/J (*A*) or C3H/HeJ (*B*) in five experiments. Each point represents the maximum response of a clone to three different doses of each splenic stimulator cell population. Each symbol indicates the results obtained from an individual mouse in the same experiment (○, Exp. 1; △, Exp. 2; ●, Exp. 3; ×, Exp. 4; and ◆, Exp. 5). (Exp. 2 and 3) Splenic stimulator cells were obtained from mice that had been injected with 200 μl anti-mouse IgD antiserum 24 h before mice were killed and cells were inactivated by 3,000-rad irradiation. (Exp. 1, 4, and 5) Splenic stimulator cells from untreated animals were treated with MMC as described previously. Maximum responses of BARB12 or BC3C4 to AKR/J in each experiment described (*A*) were the following: (Exp. 1) BARB12 = 126,399, BC3C4 = 91,358; (Exp. 2) BARB12 = 109,119, BC3C4 = 238,316; (Exp. 3) BARB12 = 19,358, BC3C4 = 78,816; (Exp. 4) BARB12 = 65,029, BC3C4 = 89,116; (Exp. 5) BARB12 = 23,787, BC3C4 = 62,316. Maximum responses of BC3C13 or BC3C4 to C3H/HeJ in each experiment described (*B*) were the following: (Exp. 1) BC3C13 = 110,176, BC3C4 = 83,159; (Exp. 2) BC3C13 = 163,298, BC3C4 = 267,539; (Exp. 3) BC3C13 = 91,484, BC3C4 = 71,101; (Exp. 4) BC3C13 = 113,889, BC3C4 = 82,507; (Exp. 5) BC3C13 = 35,673, BC3C4 = 51,336.

specific clone BC3C13 (Fig. 3 *B*). This number of Mls^c positive animals (52/70, 74.3%) is significantly different from the 50% predicted by a single locus model ($p < 0.001$).

Although it has been shown that there is no crossreactivity between Mls^a determinants and Mls^c determinants (20), it was conceivable that the apparent high frequency of expression of the Mls^c determinant detected by Mls^c-reactive cloned T cells in the strain combination above was influenced by expression of an undefined AKR/J gene. To rule out this possibility, a similar genetic segregation analysis was carried out by testing Mls^c expression in (C3H/HeJ × B10.BR) × B10.BR ([Mls^c × Mls^b] × Mls^b) offspring, a combination that involves only C3H/HeJ and B10.BR, and where B10.BR is the strain from which the Mls^c-specific clone BC3C13 was originated. As shown in Fig. 4, 49 of 66 backcross animals (74.2%) tested were Mls^c-positive as determined by the response of clone BC3C13. This percentage of Mls^c-positive animals is similar to the 75% positive offspring from the AKR/J × (AKR/J × C3H/HeJ)F_1 breeding or the 74.3% positive from (AKR/J × C3H/HeJ)F_1 × B10.BR.

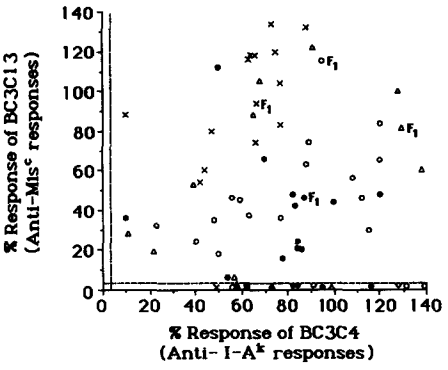

FIGURE 4. Mls^c expression by (C3H/HeJ × B10.BR) F_1 × B10.BR backcross animals. The vertical axis represents the response of Mls^c-specific clone BC3C13 and the horizontal axis represents the response of I-A^k-specific clone BC3C4 to each offspring. Each point represents the maximum response of a clone to three different doses of each splenic stimulator cell population. Each symbol indicates the results obtained from an individual mouse in the same experiment (○, Exp. 1; △, Exp. 2; ●, Exp. 3; and ×, Exp. 4). (Exp. 1, 2, and 4) Splenic stimulator cells were obtained from anti-mouse IgD-treated mice, and in Exp. 3, splenic stimulator cells from untreated animals were treated with MMC. Maximum responses of clones to C3H/HeJ in each experiment were the following: (Exp. 1) BC3C13 = 37,246, BC3C4 = 40,435; (Exp. 2) BC3C13 = 29,458, BC3C4 = 43,410; (Exp. 3) BC3C13 = 29,394, BC3C4 = 34,286; (Exp. 4) BC3C13 = 34,953, BC3C4 = 70,271.

It was recently demonstrated that the Mls^d type, which had been originally identified in the CBA/J strain, is a phenotype that results from coexpression of Mls^a and Mls^c determinants (21–23). Therefore, a segregation analysis of the Mls^c-encoding locus (or loci) was extended to the CBA/J strain. Responses of BC3C13 to splenocytes from (B10.BR × CBA/J)F_1 × B10.BR backcross animals were tested in five independent experiments and again normalized as percent responses compared with responses to C3H/HeJ splenocytes. As shown in Fig. 5, the response pattern of Mls^c-specific clone BC3C13 to CBA/J backcross animals was strikingly different from that to C3H/HeJ backcross animals. 22 of 43 backcross offspring (51.2%) were Mls^c-positive, consistent with the conclusion that the Mls^c determinant in CBA/J is controlled by a single locus.

Two Unlinked Gene Loci Encode a Common Mls^c Epitope Expressed by C3H/HeJ. The genetic control of Mls^c expression, as evaluated in the segregation studies above, was statistically analyzed. The number of Mls^c-positive offspring was determined

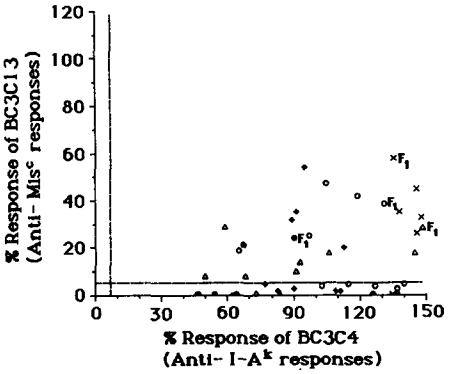

FIGURE 5. Mls^c expression by (B10.BR × CBA/J)F_1 × B10.BR backcross animals. The vertical axis represents the response of Mls^c-specific clone BC3C13 and the horizontal axis represents the response of I-A^k-specific clone BC3C4 to each offspring. Each point represents the maximum response of a clone to three different doses of each splenic stimulator cell population. Each symbol indicates the results obtained from an individual mouse in the same experiment (○, Exp. 1; △, Exp. 2; ●, Exp. 3; ×, Exp. 4; and ◆, Exp. 5). (Exp. 1, 2, and 4) Splenic stimulator cells were obtained from anti-mouse IgD-treated mice; (Exp. 3 and 5) splenic stimulator cells from untreated animals were treated with MMC. Maximum responses of clones to C3H/HeJ in each experiment were the following: (Exp. 1) BC3C13 = 23,649, BC3C4 = 27,912; (Exp. 2) BC3C13 = 139,310, BC3C4 = 26,592; (Exp. 3) BC3C13 = 52,801, BC3C4 = 33,815; (Exp. 4) BC3C13 = 11,044, BC3C4 = 27,537; (Exp. 5) BC3C13 = 43,273, BC3C4 = 30,909.

by statistical comparisons of Mls^c-specific T cell clone BC3C13 responses to experimental stimulator cells and to syngeneic B10.BR stimulator cells. This analysis is summarized in Table II. In each of the three C3H/HeJ breedings, the results of χ^2 analysis rule out the hypothesis that a single locus encodes the Mls^c determinant(s). Instead, these results were consistent with the conclusion that two unlinked loci exist, either one of which is sufficient to encode Mls^c expression. The concordant response patterns of three different Mls^c-specific clones to individual offspring indicated that the Mls^c that is segregating in this fashion does not represent multiple determinants but a single common determinant recognized by Mls^c-reactive T cells. Therefore, the most straightforward interpretation of the finding that 75% of C3H/HeJ F_2 animals are Mls^c-positive is that two unlinked genes in C3H/HeJ encode a common epitope recognized by Mls^c-specific T cell responses; the product of either of these genes is sufficient to stimulate Mls^c-reactive T cells. In contrast, although CBA/J also expresses Mls^c, only 51% of CBA/J backcross animals express Mls^c, consistent with the conclusion that unlike C3H/HeJ, the Mls^c determinant expressed in CBA/J is controlled by a single gene.

Expression of Vβ3 TCR by Peripheral T Cells Is Controlled by a Single Gene in CBA/J but by Multiple Genes in C3H/HeJ. It has recently been shown that Mls^c reactivity of T cells is strongly associated with Vβ3 TCR expression (18) and that Vβ3-expressing T cells are diminished in several Mls^c-positive strains (16, 17). Experiments were therefore designed to determine whether the two unlinked genes that encode stimulatory Mls^c determinants are also involved in Vβ3 deletion.

The relationship between Mls^c stimulation and Vβ3 expression was examined in the offspring of C3H/HeJ and CBA/J backcrosses. Vβ3$^+$ splenic T cells were identified in FCM by staining with Vβ3 TCR-specific antibody (KJ25) (16). As shown in Fig. 6, ~5% of B10.BR (Mls^b) splenic T cells were positive for Vβ3 TCR,

TABLE II

Segregation Analysis of the Gene(s) Encoding Mls^c Stimulatory Activity

		Number of Mls^c-positive animals*			
			Expected		
Offspring derived from:	Total	Observed	One locus	Two loci	Three loci
AKR/J × (AKR/J × C3H/HeJ)F_1	24	18	12 $\chi^2 = 6.0$ $p < 0.025$	19 $\chi^2 = 0.0$ $p = 1$	21 $\chi^2 = 3.43$ $0.05 < p < 0.10$
(AKR/J × C3H/HeJ)F_1 × B10.BR	70	52	35 $\chi^2 = 16.5$ $p < 0.0005$	52.5 $\chi^2 = 0.014$ $p > 0.90$	61.3 $\chi^2 = 11.35$ $p < 0.001$
(C3H/HeJ × B10.BR)F_1 × B10.BR	66	49	33 $\chi^2 = 15.5$ $p < 0.0005$	49.5 $\chi^2 = 0.02$ $p > 0.80$	57.8 $\chi^2 = 10.75$ $p < 0.005$
(B10.BR × CBA/J)F_1 × B10.BR	43	22	21.5 $\chi^2 = 0.024$ $p > 0.80$	32.2 $\chi^2 = 8.13$ $p < 0.005$	37.6 $\chi^2 = 51.5$ $p < 0.0005$

* Expression of Mls^c determinants was determined by the reactivity of Mls^c-specific clone BC3C13 to splenocytes from individual animals. Responses that were greater ($p < 0.025$ based on the student's t test) than responses to syngeneic stimulators were regarded as Mls^c positive.

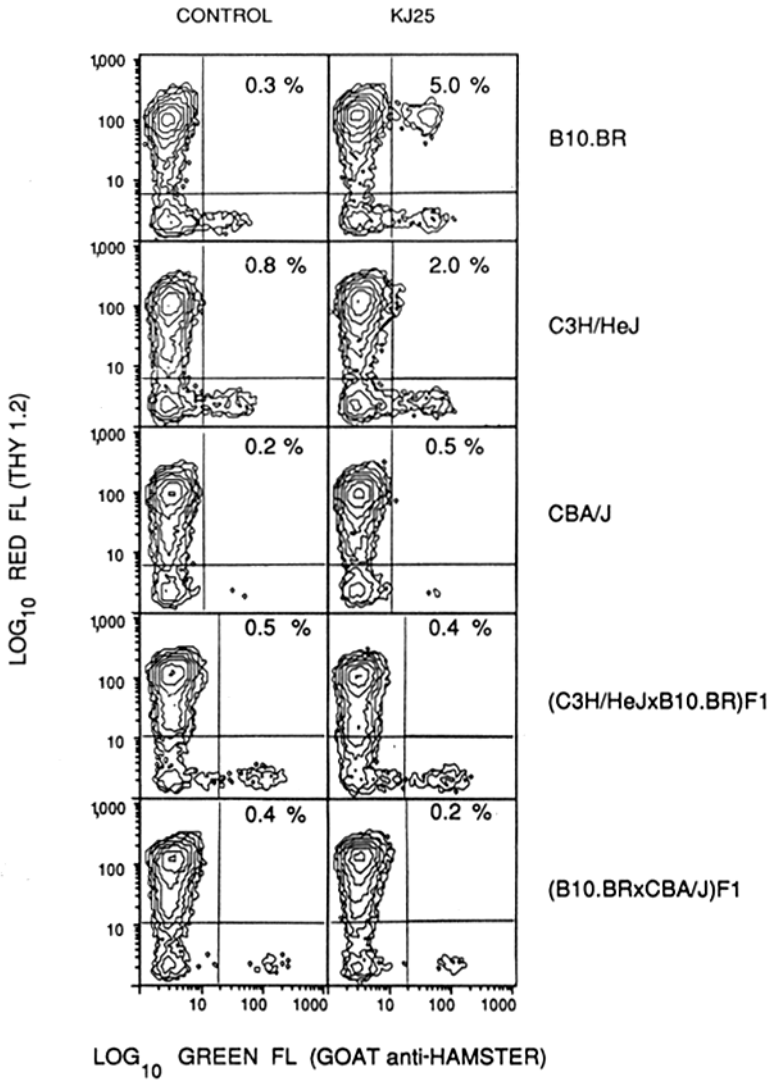

FIGURE 6. Expression of the Vβ3 TCR by splenic lymphocytes. Splenic T cells enriched by passage over rabbit anti-mouse Ig-coated plates were incubated with medium (*left column*) or with KJ25 (*right column*) culture supernatant and were stained with FITC-coupled goat anti-hamster antibody. Cells were then stained with biotin-labeled anti-Thy-1.2 antibody, counterstained with TR, and analyzed by two-color FCM. The indicated numbers are percentages of $V\beta3^+$ T cells that are calculated by dividing the number of $Thy\text{-}1^+$ $V\beta3^+$ cells by the total number of all $Thy\text{-}1^+$ cells.

whereas $V\beta3^+$ T cells were greatly reduced in C3H/HeJ or CBA/J mice, as well as their F_1 hybrids. Spleen cells from 15 (C3H/HeJ × B10.BR)F_1 × B10.BR backcross animals were tested in parallel for Mls^c expression and for Vβ3 expression. As shown in Table III, although the proportion of $V\beta3^+$ peripheral T cells varied among backcross animals, the four animals (Nos. 1, 2, 4, and 12) that were nonstimulatory for all Mls^c-reactive responder T cells used were found to express high

TABLE III

Relationship between Mls^c Stimulation and Vβ3 TCR Expression by C3H/HeJ

Stimulator*	Response of T cells‡				Mls^c phenotype§	Vβ3 expression‖
	Cloned T cells		Splenic T cells			
	BC3C13 (αMls^c)	BCAC5 (αMls^c)	AKR/J (αMls^c)	DBA/2 (αH-2^k)		
B10.BR	287¶	216	1,921	38,539	–	4.90%
C3H/HeJ	15,483	33,188	154,688	47,706	+	0.00%
(C3H/HeJ × B10.BR)F$_1$	26,571	14,943	37,387	43,437	+	0.54%
(C3H/HeJ × B10.BR)F$_1$ × B10.BR						
Backcross 1	339	291	572	45,381	–	5.80%
2	215	175	1,939	25,327	–	4.71%
3	8,482	2,378	3,933	33,204	+	0.60%
4	255	215	916	47,200	–	4.61%
5	14,320	3,365	8,741	28,019	+	0.95%
6	5,581	2,881	9,985	38,401	+	0.13%
7	2,600	459	3,814	41,245	+	0.00%
8	4,480	864	2,781	30,483	+	0.80%
9	12,855	2,925	4,346	37,659	+	0.00%
10	28,626	7,688	5,700	32,953	+	0.71%
11	14,901	2,325	3,068	31,261	+	0.32%
12	110	153	247	13,103	–	2.05%
13	4,081	667	3,405	26,931	+	0.00%
14	7,278	3,238	6,019	34,785	+	0.00%
15	6,437	3,065	8,331	34,212	+	0.00%

* Splenic stimulator cells were obtained from mice that had been injected with 200 μl goat anti-mouse IgD antiserum 24 h before mice were killed. Cells were inactivated by 3,000-rad irradiation, and 5 × 10^5 cells were added to each culture.

‡ 3 × 10^5 unprimed splenic T cells or 10^4 cloned T cells were cultured with stimulator cells.

§ Typing of Mls was based on the experimental results shown.

‖ Percentage of the Vβ3$^+$ cells in Thy-1$^+$ spleen cells, calculated by subtracting the value obtained for the control-stained cells from that obtained for the KJ25-stained cells (see Fig. 6).

¶ Arithmetic mean of [^{3}H]TdR incorporation of triplicate cultures. Underscored ($p < 0.01$) or dotted-line ($p < 0.025$) numbers are those that are significantly greater than responses to syngeneic stimulators based on the student's t test.

numbers of Vβ3$^+$ splenic T cells. All other backcross animals had <1% Vβ3$^+$ T cells, and splenic stimulator cells from those animals evoked Mls^c-specific primary as well as clone responses with the exception of Nos. 8 and 11, which stimulated responses by clone but not unprimed T cells. The relationship of Vβ3 expression and Mls^c expression was also evaluated in CBA/J by testing (B10.BR × CBA/J)F$_1$ × B10.BR backcross animals. Seven Mls^c stimulatory offspring expressed Vβ3 on <1% of splenic T cells, whereas five Mls^c-negative offspring expressed Vβ3 on 2.9–5% of T cells (Table IV). A more extensive segregation analysis of Vβ3 expression indicated that in a CBA/J-derived backcross, approximately half (14/27) of the offspring expressed high proportions of Vβ3$^+$ splenic T cells comparable with the levels expressed by the Mls^b strain B10.BR. In contrast, only 19% (7/36) of C3H/HeJ backcross mice expressed high levels of Vβ3 within 2 SD of the levels expressed by B10.BR. These results are consistent with the idea that each of the two unlinked genes controlling Mls^c expression encodes a ligand that can function in

TABLE IV

Relationship between Mls^c Stimulation and Vβ3 TCR Expression by CBA/J Backcross Splenocytes

Stimulator*	Response of T cells‡				Mls^c phenotype§	Vβ3 expression‖
	Cloned T cells		Splenic T cells			
	BC3C13 (αMls^c)	BCAC5 (αMls^c)	AKR/J (αMls^c)	DBA/2 (αH-2^k)		
B10.BR	239¶	142	1,356	18,239	–	4.27%
C3H/HeJ	29,631	23,377	56,118	22,975	+	0.00%
CBA/J	34,639	11,707	15,407	32,315	+	0.00%
(B10.BR × CBA/J)F$_1$	14,394	633	1,422	18,237	+	0.70%
(B10.BR × CBA/J)F$_1$ × B10.BR						
Backcross 1	15,064	844	4,115	14,135	+	0.00%
2	14,287	960	3,302	12,354	+	0.39%
3	12,641	2,190	7,668	15,369	+	0.53%
4	148	131	1,389	15,500	–	3.77%
5	9,205	897	2,160	13,605	+	0.00%
6	189	179	868	14,909	–	4.60%
7	256	207	842	18,025	–	4.73%
8	19,112	506	1,841	15,805	+	0.71%
9	137	163	1,009	19,601	–	2.90%
10	211	123	413	10,325	–	5.00%
11	13,595	1,895	3,783	18,561	+	0.12%
12	5,930	296	2,452	13,637	+	0.21%

* Splenic stimulator cells were obtained from mice that had been injected with 200 μl goat anti-mouse IgD antiserum 24 h before mice were killed. Cells were inactivated by 3,000-rad irradiation and 5 × 10^5 cells were added to each culture.

‡ 3 × 10^5 unprimed splenic T cells or 10^4 cloned T cells were cultured with the stimulator cells.

§ Typing of Mls was based on the experimental results shown.

‖ See Table II.

¶ See Table II.

the deletion of self-Mls^c-reactive Vβ3$^+$ T cells and that C3H/HeJ expresses both of these genes, whereas CBA/J expresses only one gene.

Discussion

In a number of instances, multigene control of immunologically relevant products has been demonstrated. In most of these cases, the product of a single gene is insufficient to generate a functional product, and the distinct products of two different genes must complement one another. Examples of this type include the two-chain structure of T and B cell antigen receptors, where combinatorial diversity is an important feature of the immune repertoire, and the polymorphism of class II MHC antigens, which is enhanced by the ability to pair different combinations of α and β chains. The results presented in this work indicate that Mls^c determinants recognized by T cells are also controlled by multiple loci. However, the nature of this genetic control is quite unique compared with the other examples previously documented. For the Mls^c strain C3H/HeJ, ~75% of Mls^a × (Mls^a × Mls^c)F$_1$, (Mls^a × Mls^c)F$_1$ × Mls^b, or (Mls^c × Mls^b)F$_1$ × Mls^b offspring expressed an apparently identical Mls^c stimulatory determinant. These results indicate that two unlinked genes encode the Mls^c stimulatory determinant in C3H/HeJ and that either

one of these genes is sufficient to encode an Mls^c determinant that is stimulatory to cloned as well as heterogeneous T cells. In contrast, only 50% of (B10.BR × CBA/J)F_1 × B10.BR backcross animals were Mls^c positive, indicating that the Mls^c determinant expressed by CBA/J is determined by a single gene. In this context, the widely scattered stimulatory potentials of segregants from C3H/HeJ may reflect the behavior of mice that express one, the other, or both Mls^c-encoding genes. As expected, the stimulatory ability of CBA/J segregants, expressing only one Mls^c gene, is more uniform.

The multigene control of the Mls^c determinant is also manifested in the striking influence of Mls gene products on the TCR Vβ repertoire. A strong association has been demonstrated between Vβ3 TCR expression and Mls^c reactivity (18), and this association is reflected in deletion of Vβ3-expressing T cells in Mls^c stimulatory strains (16, 17). In the present study, C3H/HeJ or CBA/J backcross animals that were determined to be Mls^c positive as reflected in clone or primary T cell responses were shown to express low frequencies of Vβ3$^+$ TCR in spleen cells, consistent with the conclusion that Vβ3 expression is reduced in Mls^c-positive strains. Moreover, segregation studies indicated that Vβ3 deletion in CBA/J was determined by a single gene, whereas Vβ3 deletion in C3H/HeJ could be mediated by either one of at least two unlinked genes. These results are consistent with the previous published observation by Pullen et al. (24) that two loci (one linked to MTV-13 on chromosome 4 and the other linked to Ly-7) control Vβ3 expression in DBA/2 (Mls^{a,c}) (15) or C3H/HeJ.

Click and Adelmann (25) have also concluded that there is multigene control of Mls^c expression. However, their conclusion is quite different from that described above. They concluded that four distinct Mls^c determinants were detectable using cultured cell lines obtained from different combinations of H-2^k-compatible inbred strains and that C3H/HeJ has three of four Mls^c determinants. However, the specificities of the uncloned T cell lines that were used in those studies were not confirmed to be for Mls^c determinants as defined in primary MLR.

Since it has been shown that Mls^a and Mls^c determinants are encoded by nonallelic, unlinked loci based on segregation analysis (22), a new nomenclature has been proposed in which Mls-1 would represent the locus encoding Mls^a and Mls-2 would encode what was previously known as Mls^c (26–28). The findings described above indicate the existence of a third locus (Mls-3) that is involved in Mls^c expression (25, 29). However, since the loci encoding Mls^c determinants have not yet been mapped, and since it appears that two loci may encode a common determinant, we would suggest that Festenstein's original nomenclature (9) be maintained for Mls determinants as well as for Mls phenotypes Mls^a, Mls^b, and Mls^c; Mls^d is now widely accepted to represent coexpression of Mls^a and Mls^c determinants and can be discarded from use. Thus, AKR/J would be Mls^a (instead of Mls-1^a, Mls-2^b, and Mls-3^b), B10.BR would be Mls^b (instead of Mls-1^b, Mls-2^b, and Mls-3^b), and C3H/HeJ would be Mls^c (instead of Mls-1^b, Mls-2^a, and Mls-3^a). The current ambiguity of the proposed new nomenclature reflects the fact that Mls determinants cannot be unequivocally assigned to specific loci and is most evident for CBA/J that could be genotypically (Mls-1^a, Mls-2^a, Mls-3^b), or (Mls-1^a, Mls-2^b, Mls-3^a), or even (Mls-1^a, Mls-2^b, Mls-3^b) (since it is not known whether the locus encoding Mls^c in CBA/J corresponds to either one of those expressed in C3H/HeJ).

The structural identity of Mls products is unknown. This issue can be considered in light of the finding presented here that each of two unlinked genes can encode an indistinguishable determinant that provides Mls^c stimulation to naive T cells at high precursor frequency as well as to monospecific T cell clones. One explanation of this observation is that the products encoded by the two Mls^c genes have a high degree of overall structural homology. Precedents exist, for example, in members of the Ig supergene family that are homologous to one other at the level of DNA sequence as well as molecular structure (30). The Mls^c system might be another such example. Since the Mls^c gene products encoded by these unlinked loci are as yet not distinguishable, the homology between gene products might be very high. An alternative explanation is that the two loci encode very different molecules but that the epitope recognized by T cells as Mls^c is common to the two gene products. Recent proposals that Mls gene products might be presented by APC as peptides in conjunction with MHC gene products (13, 31) are very consistent with this latter model. Molecular or biochemical analysis of the Mls^c genes or gene products will ultimately identify the correct model from these alternative possibilities.

Recent analysis has indicated that very few strains fail to express Mls^c (26, 32), indicating that the genes encoding Mls^c determinants are conserved in the mouse species. It has been also found that in Mls^c-positive strains, potentially self Mls^c-reactive Vβ3-expressing T cells are substantially deleted (16, 17). The findings presented here that multiple genes can encode the same Mls^c determinant provide another factor accounting for the wide distribution of Mls^c determinants among mouse strains. Together, these findings suggest a possible explanation for the fact that Mls-like determinants have to date been observed only in the mouse and not in any other species. If Mls-like genes and determinants in fact do exist and serve certain functions in other species such as man, the failure to identify them might be due to the difficulty in finding an Mls^- individual who would be a responder capable of responding to these determinants as alloantigens. The conserved nature of Mls-like determinants, perhaps reflecting an important biological function, would lead to difficulty in identifying such determinants on the basis of alloreactivity.

Summary

The identity of the self determinants involved in the selection of the T cell repertoire has been a matter of considerable interest. In addition to the apparent critical role of MHC gene products, accumulated experimental results indicate the importance of non-MHC gene products in T cell repertoire selection. In particular, murine Mls^a and Mls^c determinants have been shown to be highly stimulatory to allogeneic T cells and to be involved in the negative selection (elimination) of self-reactive T cells expressing selected TCR Vβ segments. In this work, a unique phenomenon of genetic redundancy is described in the control of Mls^c expression: Mls^c appears to be controlled by at least two unlinked loci, and the product of either one of these loci is sufficient to evoke Mls^c-specific T cell response and to act as a ligand in the deletion of self Mls^c-reactive V$\beta3^+$ T cells. Based on these findings, we propose a possible explanation for the fact that Mls-like genes or gene products have not been identified in other species such as man.

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References

1. Sprent, J., and S. R. Webb. 1987. Function and specificity of T cell subsets in the mouse. *Adv. Immunol.* 41:39.
2. Marrack, P., and W. K. Kappler. 1988. The T-cell repertoire for antigen and MHC. *Immunol. Today.* 9:308.
3. Matzinger, P., and M. J. Bevan. 1977. Why do so many lymphocytes respond to major histocompatibility antigens? *Cell. Immunol.* 29:1.
4. Singer, A., T. Mizuochi, T. I. Munitz, and R. E. Gress. 1986. Role of self antigens in the selection of the developing T cell repertoire. *In* Progress in Immunology VI. B. Cinader and R. G. Miller, editors. Academic Press, New York. 60–66.
5. Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature (Lond.).* 329:506.
6. Kappler, J. W., T. Wade, J. White, W. Kushnir, M. Blackman, J. Bill, N. Roehm, and P. Marrack. 1987. A T cell receptor Vβ segment that imparts reactivity to a class II major histocompatibility complex product. *Cell.* 49:263.
7. Marrack, P., and J. W. Kappler. 1988. T cells can distinguish between allogeneic major histocompatibility complex products on different cell types. *Nature (Lond.).* 332:6167.
8. Bill, J., O. Kanagawa, D. L. Woodland, and E. Palmer. 1989. The MHC molecule I-E is necessary but not sufficient for the clonal deletion of Vβ11 bearing T cells. *J. Exp. Med.* 169:1405.
9. Festenstein, H. 1973. Immunogenetic and biological aspects of in vitro lymphocyte allotransformation (MLR) in the mouse. *Transplant. Rev.* 15:62.
10. Janeway, C. A., Jr., E. A. Lerner, J. M. Jason, and B. Jones. 1980. T lymphocytes responding to Mls-locus antigens are Lyt-1$^+$, 2$^-$ and I-A restricted. *Immunogenetics.* 10:481.
11. Morrissey, P. J., D. Bradley, S. O. Sharrow, and A. Singer. 1983. T cell tolerance to non-H-2-encoded stimulatory alloantigens is induced intrathymically but not prethymically. *J. Exp. Med.* 158:365.
12. Macphail, S., S. T. Ishizaka, M. J. Bykowsky, E. C. Lattime, and O. Stutman. 1985. Specific neonatally induced tolerance to Mls locus determinants. *J. Immunol.* 135:2967.
13. Kappler, J. W., U. Staerz, J. White, and P. C. Marrack. 1988. T cell receptor Vβ elements which recognize Mls-modified products of the major histocompatibility complex. *Nature (Lond.).* 332:35.
14. MacDonald, J. R., R. Schneider, R. K. Lees, R. C. Howe, H. Acha-Orba, H. Festenstein, R. M. Zinkernagel, and H. Hengartner. 1988. T-cell receptor Vβ use predicts reactivity and tolerance to Mls^a-encoded antigens. *Nature (Lond.).* 332:40.
15. MacDonald, R. B., T. Pedrazzini, R. Schneider, J. A. Louis, R. M. Zinkernagel, and H. Hengartner. 1988. Intrathymic elimination of Mls^a-reactive (Vβ6$^+$) cells during neonatal tolerance induction to Mls^a-encoded antigens. *J. Exp. Med.* 167:2005.
16. Pullen, A. M., P. Marrack, and J. W. Kappler. 1988. The T cell repertoire is heavily influenced by tolerance to polymorphic self antigens. *Nature (Lond.).* 335:796.
17. Fry, A. M., and L. A. Matis. 1988. Self-tolerance alters T-cell receptor expression in an antigen-specific MHC restricted immune response. *Nature (Lond.).* 335:830.

18. Abe, R., M. S. Vacchio, B. Fox, and R. J. Hodes. 1988. Preferential expression of the T-cell receptor Vβ3 gene by Mls^c reactive T cells. *Nature (Lond.).* 335:827.
19. Finkelman, F. D., S. W. Kessler, J. F. Mushinski, and M. Potter. 1981. IgD-secreting murine plasmacytomas. Identification and potential characterization of two IgD myeloma proteins. *J. Immunol.* 126:680.
20. Abe, R., J. J. Ryan, F. D. Finkelman, and R. J. Hodes. 1987. T cell recognition of Mls: T cell clones demonstrate polymorphism between Mls^a, Mls^c and Mls^d. *J. Immunol.* 138:373.
21. Abe, R., J. J. Ryan, and R. J. Hodes. 1987. Clonal analysis of the Mls system. A reappraisal of polymorphism and allelism among Mls^a, Mls^c, and Mls^d. *J. Exp. Med.* 165:1113.
22. Abe, R., J. J. Ryan, and R. J. Hodes. 1987. Mls is not a single gene, allelic system. Different stimulatory Mls determinants are the products of at least two nonallelic, unlinked genes. *J. Exp. Med.* 166:1150.
23. Ryan, J. J., J. J. Mond, and F. D. Finkelman. 1987. The Mls^d-defined primary mixed lymphocyte reaction: a composite response to Mls^a and Mls^c determinants. *J. Immunol.* 138:4385.
24. Pullen, A. M., P. Marrack, and J. W. Kappler. 1989. Evidence that Mls-2 antigens which delete Vβ3$^+$ T cells are controlled by multiple genes. *J. Immunol.* 142:3033.
25. Click, R. E., and A. Adelmann. 1988. Multi-gene control of Mls^c. *Immunogenetics.* 28:412.
26. Abromson-Leeman, S. R., J. C. Lanig, and M. E. Dorf. 1988. T cell recognition of Mls^{c,x} determinants. *J. Immunol.* 140:1726.
27. Janeway, C. A., Jr., J. Yagi, P. J. Conrad, M. E. Katz, B. Jones, S. Vroegop, and S. Buxser. 1989. T cell responses to Mls and to bacterial proteins that mimic its behavior. *Immunol. Rev.* 107:60.
28. Abe, R., and R. J. Hodes. 1989. The Mls system; non-MHC genes that encode strong T cell stimulatory determinants. *Immunol. Today.* 9:230.
29. Abe, R., and R. J. Hodes. 1989. Properties of the Mls system: a revised formulation of Mls genetics and an analysis of T-cell recognition of Mls determinants. *Immunol. Rev.* 107:6.
30. Kronenberg, M., G. Siu, L. E. Hood, and N. Shastri. 1986. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. *Annu. Rev. Immunol.* 4:529.
31. Dekruyff, R. H., S. Ju, J. Laning, H. Cantor, and M. E. Dorf. 1986. Activation requirements of cloned inducer T cells. III. Need for two stimulator cells in the response of a cloned line to Mls determinants. *J. Immunol.* 137:1109.
32. Abe, R., and R. J. Hodes. 1989. T-cell recognition of minor lymphocyte stimulating (Mls) gene products. *Annu. Rev. Immunol.* 7:683.