CLONAL ANALYSIS OF THE Mls SYSTEM

A Reappraisal of Polymorphism and Allelism among Mls^a, Mls^c, and Mls^d

By RYO ABE, JOHN J. RYAN,* AND RICHARD J. HODES

*From the Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, and the *Transplantation Research Program Center, Naval Medical Research Institute, Bethesda, Maryland 20814*

Two sets of antigenic stimuli are recognized by naive T cell populations at a sufficiently high precursor frequency to induce primary proliferative responses. One of these, the set of MHC-encoded gene products (1, 2), has been well characterized both structurally and functionally (3, 4), and clearly plays a central role in immune recognition. In contrast, despite the extraordinarily high frequency of Mls^a- or Mls^d-reactive T cells in both naive (5–8) and cloned T cell populations (9, 10), the structural and functional characterization of the set of minor lymphocyte stimulating (Mls) gene products has proven to be far more difficult. One area of continued uncertainty concerns the nature of the polymorphism in expressed Mls determinants. The Mls locus was originally described as having four alleles, Mls^a, Mls^b, Mls^c, and Mls^d, that encode polymorphic determinants recognized by T cells (5). These determinants have widely differing stimulatory capacities: Mls^a and Mls^d are strongly stimulatory, Mls^c is intermediate, and Mls^b is nonstimulatory. Additional findings have more recently been interpreted to suggest that the products of Mls genes a, b, c, and d express unique variable determinants specific for each allele (11), and the existence of another stimulatory Mls type, Mls^x, has been reported (12). In contrast, however, based on recent observations, several investigators have raised questions concerning the polymorphism of Mls. In addition to the difficulties in analyzing Mls^b and Mls^c because of the absence or weakness, respectively, of their stimulatory effects, one controversial point has concerned the nature of the strongly stimulatory Mls^d. Although Mls^d was originally identified on CBA/J as an independent allele of the Mls system, it has been reported by several investigators that Mls^a and Mls^d are highly crossreactive (13–16) and even antigenically indistinguishable (14). Based on such findings, it has been suggested that the Mls locus has only two alleles, the a/d allele causing strong mixed lymphocyte responses (MLR) and the null b allele (15); or alternatively, that the Mls locus encodes nonpolymorphic cell surface determinants that might determine the Mls type quantitatively, a and d expressing higher amounts of these determinants than do c and b (17). Inasmuch as Mls determinants cannot at present be detected serologically but

J. J. Ryan was supported by Naval Medical Research and Development Command work unit M0095.001.1055. Address correspondence to Dr. Ryo Abe, National Institutes of Health, Building 10, Room 4B17, Bethesda, MD 20892.

only as lymphocyte-activating determinants in MLR among MHC-identical inbred strains, the resolution of the issue of Mls polymorphisms is not yet clear.

In a previous report (18), we demonstrated that Mls^a-specific T cell clones responded to Mls^a (but not to Mls^c or Mls^b) stimulators and that Mls^c-specific clones responded to Mls^c, but not Mls^a or Mls^b, indicating that polymorphism does exist at least between Mls^a and Mls^c. In those studies, it was also observed that CBA/J (Mls^d) stimulators could stimulate both Mls^a- and Mls^c-specific clones, suggesting that the product(s) expressed by Mls^d might consist of components expressed by Mls^a and Mls^c antigens. The present study represents a clonal analysis of the relationship between anti-Mls^a, anti-Mls^c, and anti-Mls^d responses. The findings presented here further characterize the nature of polymorphism within the Mls system, suggest that the Mls^a and Mls^c genes are not allelic, and indicate that the Mls^d phenotype represents the coexpression of Mls^a and Mls^c.

Materials and Methods

Mice. A/J, AKR/J, B10.A, B10.BR, B10.D2, C57L/J, C3H/HeJ, CBA/J, D1.LP, DBA/2, B × D recombinant inbred (RI)[1] and B × H RI mice were purchased from The Jackson Laboratory, Bar Harbor, ME. (AKR/J × C3H/HeJ)F_1 and (CBA/J × B10.BR)F_1 mice as well as AKR/J × (AKR/J × C3H/HeJ) and (CBA/J × B10.BR) × B10.BR backcross mice were bred in our own facilities.

Culture Medium. RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 5×10^{-5} M 2-ME, and 10% FCS was used for T cell proliferation assays and maintenance of T cell clones.

Antibody. Goat anti–mouse IgD antibody was kindly provided by Dr. Fred D. Finkelman (Uniformed Services University of the Health Sciences, Bethesda, MD). The generation of this antibody has been described elsewhere (19).

Derivation of T Cell Lines and Clones. Nylon wool nonadherent T cells (NNT) were isolated from unprimed B10.BR spleen cells and treated with anti-Lyt-2.2 (No. FPA-179; New England Nuclear Boston, MA) (final concentration 1:3,200) and complement. 5×10^5 Lyt-2^- NNT were cultured in the presence of 5×10^5 CBA/J spleen cells which were inactivated by treatment with 50 μg/ml mitomycin C (MMC; Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C. The cells were restimulated every 10 d as above. At the end of each cycle, the cells were layered and centrifuged over Lymphocyte Separation Medium (Litton Bionetics, Inc., Charleston, SC) to remove dead cells. After four cycles of stimulation, the T cell lines were cloned by limiting dilution in the presence of 10% lectin-free culture supernatant from Con A–stimulated BALB/c spleen cells as a source of IL-2. The clones were stimulated every 10 d in the presence of MMC-treated CBA/J spleen cells and 50 U/ml of human recombinant IL-2 (kindly supplied by the Cetus Corp., Emeryville, CA).

T Cell Proliferation Assay. T cell proliferation was assayed by culturing either 3×10^5 unprimed NNT or 10^4 cloned T cells with 3×10^5 unprimed spleen cells inactivated by 2,500 rad irradiation or MMC treatment in a total volume of 200 μl of complete medium containing 10% FCS in flat-bottomed wells. After incubating for 4 d at 37°C in 5% CO_2 humidified air, the amount of [^{3}H]thymidine incorporated during a 12 h pulse (1 μCi/well, sp act 2 Ci/mM; New England Nuclear, Boston, MA) was assessed. Results are expressed as the arithmetic means of triplicate cultures. Standard errors were generally <10% of the mean.

In Vivo Anti-IgD–treated Stimulator Cells. Since it had been reported that the capacity of splenocytes to stimulate across an Mls difference was enhanced after exposure to anti-IgD antibody without altering the specificity of the response (20), in some experiments,

[1] *Abbreviations used in this paper:* MMC, mitomycin C; NNT, nylon wool nonadherent T cells; RI, recombinant inbred.

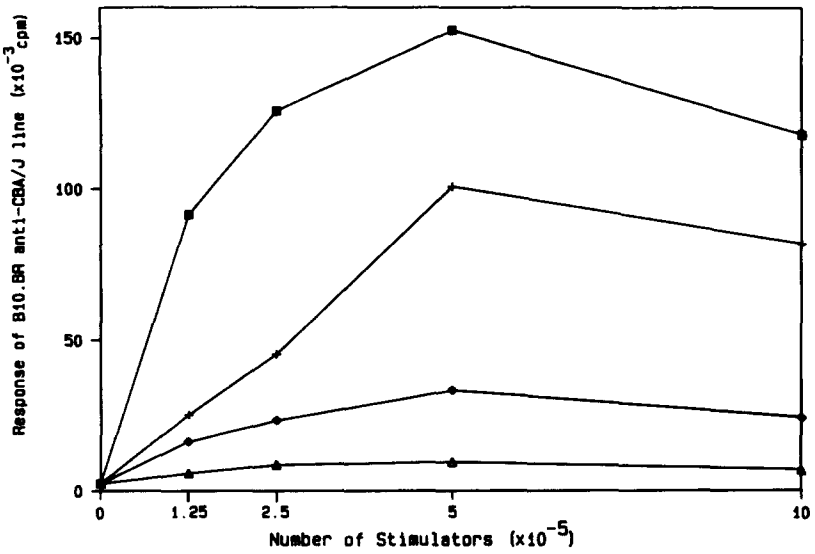

FIGURE 1. Responses of a B10.BR anti-CBA/J T cell line to MHC-identical, Mls-disparate stimulators. 10^4 T cells were cultured with varying numbers of MMC-treated splenic stimulator cells from CBA/J (H-2^k, Mls^d) (■), AKR/J (H-2^k, Mls^a) (+), C3H/HeJ (H-2^k, Mls^c) (◇), or B10.BR (H-2^k, Mls^b) (△). Each point represents the mean of [^{3}H]TdR incorporation of triplicate cultures. Incorporation by T cells cultured alone and by each dose of stimulator cells cultured alone was <1,000 cpm.

200 μl of goat anti-IgD antiserum was injected 24 h before mice were killed as a source of stimulator cells. The spleens were removed and gently teased in RPMI 1640 supplemented with FCS, washed three times, and inactivated by irradiation or MMC treatment. The response patterns of unprimed B10.BR T cells stimulated with anti-IgD–treated or untreated stimulators from several inbred strains were found to be similar in repeated experiments.

Results

Generation of B10.BR Anti-CBA/J T Cell Clones. To clarify the nature of the anti-Mls^d response and its relation to anti-Mls^a and anti-Mls^c responses, an attempt was made to carry out a clonal analysis of the T cells responding to Mls^d stimulators. Unprimed Lyt-2$^-$ B10.BR (H-2^k, Mls^b) NNT cells were allowed to respond to MMC-treated and T cell–depleted CBA/J (H-2^k, Mls^d) spleen cells in a primary MLR. After four cycles of stimulation, cells were tested for their reactivity to H-2-identical, Mls-disparate stimulators. As shown in Fig. 1, these T cells responded strongly to the original stimulator strain, CBA/J (Mls^d). In addition, these cells were stimulated by AKR/J (Mls^a) and C3H/HeJ (Mls^c) but only weakly by syngeneic B10.BR stimulators (relative to ^{3}H uptake by responders alone plus uptake by stimulators alone). These results suggested that this continuous T cell line contained Mls^d-, Mls^a-, and Mls^c-reactive T cell clones. Therefore, this line was cloned by limiting dilution at 0.3 cells/well. Nine clones that grew well were tested for their ability to respond to four H-2-identical, Mls-different strains, AKR/J (Mls^a), B10.BR (Mls^b), C3H/HeJ (Mls^c) and CBA/J (Mls^d) (Table I). Each of these clones responded to CBA/J stimulators but not syngeneic B10.BR stimulators, indicating that these clones were not self MHC–reactive. In

TABLE I

Responses of Clones from a B10.BR Anti-CBA/J Line to Stimulator Cells from Mls-different Strains

T cell clones	Response of T cell clones* with stimulator cells from:‡				
	None	CBA/J (Mls^d)	B10.BR (Mls^b)	AKR/J (Mls^a)	C3H/HeJ (Mls^c)
—	—	1,651§	1,482	477	881
BCAC1	841	33,825	861	469	33,049
BCAC2	621	6,532	596	5,428	477
BCAC3	1,052	24,257	739	14,282	313
BCAC4	1,331	24,825	1,105	26,013	841
BCAC5	658	40,191	408	809	36,835
BCAC6	709	14,819	1,048	19,565	1,028
BCAC10	598	26,915	945	25,793	898
BCAC12	936	48,216	446	40,597	931
BCAC17	1,419	49,661	1,028	24,292	838

* 10^4 cloned T cells were cultured with the stimulator cells.

‡ 3×10^5 MMC-treated stimulator cells were added to each culture.

§ Arithmetic mean of [^{3}H]TdR incorporation of triplicate cultures. Underlined numbers are those that are significantly greater ($p < 0.05$) than responses to syngeneic (B10.BR) stimulators.

addition to their reactivity to CBA/J, each of these clones had a second reactivity to Mls-different stimulators; seven clones responded to Mls^a (AKR/J) and two clones responded to Mls^c (C3H/HeJ) cells. None of these clones reacted with both AKR/J and C3H/HeJ stimulators. This pattern of responsiveness of anti-CBA/J clones to stimulators of other Mls types suggested the possibility that T cells responding to Mls^d determinants crossreacted with Mls^a or Mls^c in a clonally distinct manner. Alternatively, however, since AKR/J and C3H/HeJ cells differ in the expression of multiple non-MHC products, these clones might have been specific for minor antigens distinct from Mls^a or Mls^c. To evaluate these possibilities, the specificities of these clones were further examined.

The Specificity of Crossreactivity to AKR/J Stimulators by Anti-CBA/J Clones Is Anti-Mls^a. To determine the crossreactive specificity of those anti-CBA/J clones that were reactive to AKR/J stimulators, these clones were examined for their reactivities to various H-2-, background-, and Mls-different stimulators. The responses of two representative clones are shown in Table II. It is apparent that each of these clones was able to respond to Mls^d (CBA/J) and Mls^a (AKR/J, DBA/2, D1.LP) but not to Mls^b (B10.BR, B10.D2, C57L/J) or Mls^c (C3H/HeJ) cells. Since the Mls^a gene has already been mapped by the use of RI strains (6), such mice were used to determine the precise specificity of the T cell clones. Two clones, BCAC3 and BCAC4, were cultured with spleen cells from each of 20 B × D RI strains (Table III). All 11 strains of the Mls^a genotype stimulated each clone, but none of the 9 Mls^b strains was stimulatory. This pattern of responsiveness indicates that determinants recognized by these clones were encoded by the Mls^a gene (or a closely linked gene).

The Specificity of Crossreactivity to C3H/Hej Stimulators by Anti-CBA/J Clones is Anti-Mls^c. The specificity of two B10.BR anti-CBA/J clones that reacted with C3H/HeJ (Mls^c) stimulators was first analyzed by stimulating the clones with splenocytes from six inbred strains (Table IV). The two clones BCAC1 and

TABLE II

Response Patterns of AKR/J-reactive Clones from a B10.BR Anti-CBA/J Line

Stimulator strain*	Genotype		Response of T cell clones‡	
	H-2	Mls	BCAC3	BCAC4
AKR/J	k	a	49,746§	25,695
B10.BR	k	b	559	718
DBA/2	d	a	14,029	35,882
B10.D2	d	b	114	242
D1.LP	b	a	48,491	55,187
C57L/J	b	b	159	149
C3H/HeJ	k	c	196	220
CBA/J	k	d	69,924	42,439

* 3×10^5 MMC-treated splenic stimulator cells were added to each culture.
‡ 10^4 cells of each T cell clone were cultured with the stimulator cells.
§ Arithmetic mean of [^{3}H]TdR incorporation of triplicate cultures. Underlined numbers are those that are significantly greater ($p < 0.05$) than responses to syngeneic (B10.BR) stimulators.

TABLE III

Responses of AKR/J-reactive T Cell Clones to Stimulator Cells from B × D Recombinant Inbred Mice

B × D stimulator strain*	Genotype		Response of T cell clones‡	
	H-2	Mls	BCAC3	BCAC4
No. 1	d	b	755§	283
2	b	a	4,750	9,539
5	d	a	14,015	72,980
6	d	b	441	309
8	b	a	11,777	91,768
9	d	a	17,578	63,132
11	d	a	14,920	63,549
12	d	b	591	411
14	b	b	890	564
15	b	b	398	527
16	d	b	746	385
18	d	b	388	611
22	d	a	9,255	19,224
23	b	b	353	262
24	d	a	20,206	129,808
25	d	a	11,433	78,063
27	d	a	14,451	91,663
28	d	a	10,021	114,522
29	b	a	5,799	21,649
31	d	b	740	825

* 3×10^5 MMC-treated splenic stimulator cells were added to each culture.
‡ 10^4 cells of each T cell clone were cultured with the stimulator cells.
§ Arithmetic mean of [^{3}H]TdR incorporation of triplicate cultures. Underlined numbers are those that are significantly greater ($p < 0.05$) than responses to syngeneic (B10.BR) stimulators.

TABLE IV
Response Patterns of C3H/HeJ-reactive Clones from a B10.BR Anti-CBA/J Line

Stimulator strain*	Genotype		Response of T cell clones‡	
	H-2	Mls	BCAC1	BCAC5
C3H/HeJ	k	c	38,952§	28,437
A/J	a	c	40,931	19,293
B10.BR	k	b	383	372
B10.A	a	b	305	226
CBA/J	k	d	53,363	39,164
AKR/J	k	a	313	296

* 3×10^5 MMC-treated splenic stimulator cells were added to each culture.
‡ 10^4 cells of each T cell clone were cultured with the stimulator cells.
§ Arithmetic mean of [^{3}H]TdR incorporation of triplicate cultures. Underlined numbers are those that are significantly greater ($p < 0.05$) than responses to syngeneic (B10.BR) stimulators.

BCAC5 responded to Mls^d (CBA/J) and Mls^c (C3H/HeJ and A/J) spleen cells but not Mls^b (B10.BR, B10.A) or Mls^a (AKR/J) spleen cells. Therefore these clones were considered to be potentially Mls^c-specific. Unlike Mls^a, the Mls^c gene has not yet been formally mapped. Therefore, the only available means to determine whether T cell clones are Mls^c-specific was to compare the pattern of proliferative responses of the potential Mls^c-reactive clones with the anti-Mls^c responses of unprimed T cells to different stimulators.

The first comparison of clone and primary responses was carried out using B × H RI strains (Table V). These B × H strains were derived from an initial cross between C57BL/6J (H-2^b, Mls^b) and C3H/HeJ (H-2^k, Mls^c) and thus express a segregated distribution of the genes expressed by these two parental strains. Spleen cells from only H-2^k B × H RI strains, as well as control strains C3H/HeJ and B10.BR, were used as stimulators since H-2^b, Mls^c strains stimulate only weakly as previously reported (21). As shown in Table V, stimulator cells from B × H strains 3, 6, 12, and 14 induced strong proliferative responses both of unprimed B10.BR T cells and of the two potential Mls^c-reactive clones, whereas B × H 7 stimulated neither of these responders. These differences did not appear to reflect simply the overall condition of stimulator cells because all populations could stimulate an I-A^k-reactive clone (BC3C4). The precise correlation of the proliferative responses of these clones with the responses of unprimed B10.BR T cells to H-2-identical B × H cells suggested that these clones were Mls^c-specific.

This possibility was further examined by a second approach that compared primary Mls^c responses and cloned T cell responses using AKR/J × (AKR/J × C3H/HeJ)F$_1$ backcross animals. In the progeny of this backcross, there should be Mls$^{a/a}$ and Mls$^{a/c}$ animals. Theoretically, AKR/J (Mls^a) T cells could only be stimulated by the Mls$^{a/c}$ animals. Thus, if the Mls^c-reactive clones are in fact Mls^c-specific, they should only respond to those stimulator cells that can stimulate unprimed AKR/J T cells. Based on this hypothesis, responses of unprimed AKR/J T cells and clones to stimulator cells from backcross animals were studied (Fig. 2). Responder T cells from unprimed AKR/J (H-2^k, Mls^a); two putative Mls^c-

TABLE V

Responses of Unprimed B10.BR T Cells and C3H/HeJ-reactive Clones from a B10.BR Anti-CBA/J Line to Stimulator Cells from B × H Recombinant Inbred Mice

Stimulator strain*	H-2 genotype	Responding T cells‡			
		Anti-CBA/J clones		Anti-I-A^k	Unprimed T cells
		BCAC1	BCAC5	BC3C4§	B10.BR
B × H:					
3	k	5,007‖	11,696	101,416	10,937
6	k	12,311	26,049	75,190	17,998
7	k	246¶	364	111,275	6,839
12	k	5,323	13,219	65,640	18,156
14	k	5,341	12,959	71,093	12,463
C3H/HeJ	k	5,834	16,578	107,391	30,529
B10.BR	k	133	481	108,706	5,416

* Splenic stimulator cells were obtained from mice that were injected with 200 μl of goat anti-mouse IgD antiserum 24 h before mice were killed. Cells were treated with MMC and 3×10^5 cells were added to each culture.

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

§ Anti-I-A^k clone (18).

‖ Arithmetic mean of [^{3}H]TdR incorporation of triplicate cultures.

¶ Underlined numbers are those that are not significantly greater ($p < 0.05$) than responses to syngeneic (B10.BR) stimulators.

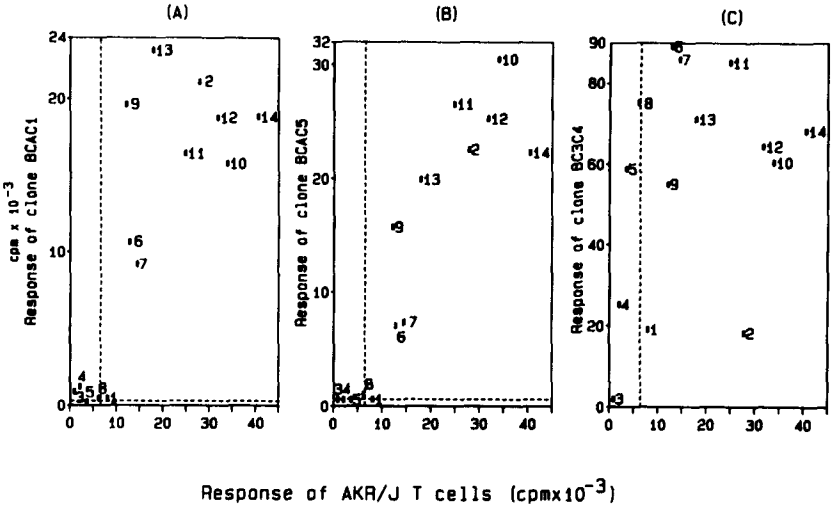

FIGURE 2. Primary and cloned T cell responses to AKR/J × (AKR/J × C3H/HeJ)F_1 backcross mice. The vertical axis represents the response of 10^4 cloned anti-CBA/J T cells, BCAC1 (*A*) and BCAC5 (*B*); or anti-I-A^k T cells, BC3C4 (*C*). Each point on the graph indicates the responses of a T cell clone and of 3×10^5 unprimed AKR/J T cells to 3×10^5 MMC-treated splenic stimulator cells from numbered individual backcross mice. Horizontal and vertical dotted lines indicate the background of responses obtained when each responder population was cultured with autologous spleen cells.

specific anti-CBA/J clones, BCAC1 (Fig. 2*A*) and BCAC5 (Fig. 2*B*); and one I-A^k-reactive clone, BC3C4 (Fig. 2*C*), were cultured with stimulator cells from individual AKR/J × (AKR/J × C3H/HeJ)F_1 backcross mice. Each numbered

TABLE VI

Proliferative Responses of B10.BR Anti-CBA/J Clones to H-2-identical, Mls-different Stimulator Cells

Stimulator strain*	Genotype		Response of T cell clones‡			
	H-2	Mls	BCAC1	BCAC3	BCAC4	BCAC5
AKR/J	k	a	380§	47,763	16,640	406
B10.BR	k	b	383	886	552	200
C3H/HeJ	k	c	32,431	298	743	17,863
CBA/J	k	d	24,419	63,293	34,379	19,962
(AKR × C3H)F_1	k/k	a/c	33,162	33,634	19,128	5,834

* 3×10^5 MMC-treated stimulator cells were added to each culture.

‡ 10^4 cloned T cells were cultured with the stimulator cells.

§ Arithmetic mean of [^{3}H]TdR incorporation of triplicate cultures. Underlined numbers are those which are significantly greater ($p < 0.05$) than responses to syngeneic (B10.BR) stimulators.

point in the figure represents counts per minute of [^{3}H]thymidine incorporation by unprimed AKR/J T cells (abscissa) and T cell clones (ordinate). Stimulator cells stimulated an I-A^k-specific clone (BC3C4), confirming their general stimulatory capacities,[2] and these responses showed no correlation with the responses of AKR/J T cells (Fig. 2*C*). Stimulator cells from backcross animals 2, 6, 7, 9, 10, 11, 12, 13, and 14 stimulated both unprimed AKR/J T cells and clones BCAC1 (Fig. 2*A*) and BCAC5 (Fig. 2*B*). In contrast, stimulator cells from animals 1, 4, 5, and 8 did not significantly stimulate AKR/J T cells or the potential Mls^c-reactive clones. Since the results from each of these approaches showed a precise correlation between response patterns of presumably Mls^c-specific unprimed T cells and potential Mls^c-reactive clones, it appears that the crossreactivity of these two anti-CBA/J clones to C3H/HeJ stimulators is Mls^c specific.

Determinants Recognized by All Anti-CBA/J Clones Are Expressed on $Mls^{a/c}$ F_1 Splenocytes. Once the crossreactive specificities of these anti-CBA/J clones were determined to be for Mls^a or Mls^c, further clonal comparisons were carried out to analyze the relationship between the determinants expressed on CBA/J (Mls^d) stimulators and those expressed on Mls^a or Mls^c stimulators. The two groups of T cell clones reactive with CBA/J cells and crossreactive to Mls^a or Mls^c were retested for their ability to proliferate in response to Mls-disparate stimulator cells as well as to $Mls^{a/c}$ F_1 [(AKR/J × C3H/HeJ)F_1] cells (Table VI). All clones responded to CBA/J stimulators, whereas Mls^a-reactive clones (BCAC3, BCAC4) uniformly responded to Mls^a but not to Mls^c cells; and Mls^c-reactive clones (BCAC1, BCAC5) responded only to Mls^c stimulator cells. These results indicated that although determinants on CBA/J cells, as recognized by T cells, are crossreactive to either Mls^a or Mls^c, there is no crossreactivity between Mls^a and Mls^c. Furthermore, since all CBA/J-reactive clones responded to (AKR/J × C3H/HeJ)F_1 cells, determinants recognized by all these clones are expressed on $Mls^{a/c}$ F_1 splenocytes.

Unidirectional Stimulation: Mls^d Cells Stimulate but Do Not Respond to Mls^a and Mls^c. The Mls^d determinants were originally defined by the ability of unprimed

[2] Stimulator cells from backcross animal 3 showed no stimulatory ability whatever and were therefore excluded from further analysis.

TABLE VII

Unidirectional Stimulation between Mls^d and Mls^a or Mls^c in Primary MLR

Stimulator strain*	Genotype		Responding T cells‡				
	H-2	Mls	AKR/J (H-2^k, Mls^a)	B10.BR (H-2^k, Mls^b)	C3H/HeJ (H-2^k, Mls^c)	CBA/J (H-2^k, Mls^d)	B10.D2 (H-2^d, Mls^b)
AKR/J	k	a	1,316§	77,976	155,055	6,885	82,174
B10.BR	k	b	1,933	3,114	3,926	8,371	25,769
C3H/HeJ	k	c	31,531	20,718	2,414	6,589	34,432
CBA/J	k	d	6,885	80,602	142,740	6,828	107,352
B10.D2	d	b	34,598	31,229	20,144	34,598	2,982

* Splenic stimulator cells were obtained from mice that were injected with 200 μl of goat anti-mouse IgD antiserum 24 h before mice were killed. Cells were treated with MMC and 3×10^5 cells were added to each culture.

‡ 3×10^5 unprimed NNT were cultured with the stimulator cells.

§ Arithmetic mean of [^{3}H]TdR incorporation of triplicate cultures. Underlined numbers are those which are significantly greater ($p < 0.05$) than responses to syngeneic stimulators.

T cells derived from nonidentical but MHC-matched strains to respond to CBA/J stimulators. Since the gene(s) encoding Mls^d determinants expressed on CBA/J splenocytes have not been mapped, it is difficult to definitively establish whether T cell clones are specific for Mls^d. Therefore, the pattern of primary T cell responses among CBA/J cells and cells of other Mls types was reevaluated to directly assess the behavior of Mls^d determinants in primary MLR. As shown in Table VII, CBA/J (Mls^d) splenocytes stimulated B10.BR (Mls^b) or C3H/HeJ (Mls^c) T cells strongly, and stimulated AKR/J (Mls^a) T cells significantly, although to a lesser degree. These results confirmed those recently reported by Ryan et al. (22; Ryan, J. J., J. J. Mond, and F. D. Finkelman, manuscript submitted for publication) and indicated that Mls^d determinants are not identical to Mls^a but include determinants that stimulate unprimed Mls^a T cells to proliferate. In contrast, although CBA/J (Mls^d) T cells responded well to H-2 antigens expressed on MHC-disparate stimulators (B10.D2), these T cells failed to respond to any H-2-compatible, Mls-disparate stimulators. These results suggested that T cells from Mls^d mice are genetically tolerant to Mls^a and Mls^c determinants, and therefore that Mls^a and Mls^c determinants are expressed in Mls^d mice.

Is the Anti-Mls^d Response Attributable to T Cell Recognition of Only Mls^a and Mls^c Determinants? Although it was shown by using a panel of B10.BR anti-CBA/J clones that Mls^d cells are recognized by Mls^a- and Mls^c-reactive T cells, this limited clonal analysis does not necessarily mean that all of the Mls^d-specific response is caused by Mls^a or Mls^c. To test the possibility that Mls^d determinants exist that are distinct from either Mls^a or Mls^c, (AKR/J × C3H/HeJ)F$_1$ (Mls$^{a/c}$) T cells were stimulated by cells of different Mls types (Table VIII). In each of two experiments, (AKR/J × C3H/HeJ)F$_1$ T cells responded to CBA/J stimulators to a degree that was marginally greater ($p < 0.05$) than the responses of F$_1$ T cells to syngeneic F$_1$ stimulators but was not greater than the responses of CBA/J T cells to CBA/J stimulators. Thus, these experiments did not clearly identify (nor did they exclude) the existence of a significant response by Mls$^{a/c}$ T cells to Mls^d stimulators.

The Mls^a- and Mls^c-like Determinants Expressed by CBA/J (Mls^d) Cells Are Encoded

TABLE VIII

Responses of $Mls^{a/c}$ F_1 T Cells to Mls^d Stimulator Cells

Stimulator strain*	Genotype		Responding T cells‡		
	H-2	Mls	CBA/J	(AKR × C3H)F_1	B10.D2
Exp. 1					
AKR/J	k	a	732§	974	134,344
C3H/HeJ	k	c	1,561	1,995	64,065
CBA/J	k	d	2,695	2,981	218,155
(AKR × C3H)F_1	k/k	a/c	1,924	1,167	118,834
B10.D2	d	b	19,740	31,112	1,448
Exp. 2					
AKR/J	k	a	4,791	1,053	115,997
C3H/HeJ	k	c	3,675	2,836	79,507
CBA/J	k	d	4,999	4,963	277,634
(AKR × C3H)F_1	k/k	a/c	3,013	2,741	274,547
B10.D2	d	b	18,023	18,931	655

* Splenic stimulator cells were obtained from mice that were injected with 200 μl of goat anti-mouse IgD antiserum 24 h before mice were killed. Cells were treated with MMC and 3×10^5 cells were added to each culture.

‡ 3×10^5 unprimed NNT were cultured with the stimulator cells.

§ Arithmetic mean of [^{3}H]TdR incorporation of triplicate cultures. Underlined numbers are those that are significantly greater ($p < 0.05$) than responses to syngeneic stimulators.

by Nonallelic and Apparently Unlinked Genes. The previously reported ability of all Mls^a- and Mls^c-specific clones to respond to CBA/J (Mls^d) stimulators (18), combined with the ability of all B10.BR anti-CBA/J clones to respond to either Mls^a or Mls^c stimulators, indicated that Mls^a- and Mls^c-like determinants are coexpressed on CBA/J cells. This coexpression could result from the existence of a single (Mls^d) gene product that expressed both Mls^a- and Mls^c-like determinants. Alternatively, coexpression could reflect the independent expression by CBA/J cells of two different genes, one of them encoding Mls^a or an Mls^a-like product and the other encoding Mls^c or an Mls^c-like product. These alternative possibilities were evaluated by testing the stimulatory capacity of spleen cells from progeny of the (CBA/J × B10.BR)F_1 × B10.BR backcross. If the Mls^a and Mls^c determinants expressed by CBA/J were encoded by the same gene (or closely linked genes), then all offspring of this $Mls^{d/b}$ × Mls^b backcross would either express both Mls^a and Mls^c determinants (the $Mls^{d/b}$ genotype) or neither Mls^a nor Mls^c (the $Mls^{b/b}$ genotype). In contrast, if unlinked CBA/J genes encoded Mls^a- and Mls^c-like determinants, then some offspring would express Mls^a and not Mls^c while others would express Mls^c and not Mls^a. A total of 37 backcross animals were tested in four different experiments, and the results from one experiment are presented in Table IX. Phenotyping of these mice was carried out using B10.BR anti-CBA/J clones that were either Mls^a-specific (BCAC3 and BCAC4) or Mls^c-specific (BCAC1 and BCAC5). Spleen cells from all 10 backcross progeny stimulated an I-A^k-specific T cell clone. Cells from some of these progeny stimulated both Mls^a-specific and Mls^c-specific clones (e.g., offspring No. 2), whereas cells from other progeny stimulated neither Mls^a-specific nor Mls^c-specific clones (Nos. 1, 3, 5, and 10). In addition, however, some offspring populations stimulated Mls^a-specific but not Mls^c-specific clones (Nos. 6 and 7),

TABLE IX
Responses of CBA/J-reactive T Cell Clones to Backcross Stimulators: (CBA/J × B10.BR)F_1 × B10.BR

Stimulator strain*	Responder T cells‡						
	AKR-reactive clones		C3H-reactive clones		Anti-I-A^k clone	Mls type	
	BCAC3	BCAC4	BCAC1	BCAC5	BC3C4§	a	c
AKR/J	41,611‖	23,195	1,157	809	41,979	+	−
C3H/HeJ	643	547	45,203	18,360	27,921	−	+
CBA/J	57,906	41,453	63,373	30,981	33,695	+	+
B10.BR	1,162	501	1,381	645	48,623	−	−
(CBA × B10.BR)F_1	35,374	30,311	8,656	5,398	32,903	+	+
(CBA × B10.BR)F_1 × B10.BR							
1	737	720	1,156	517	18,154	−	−
2	50,823	31,066	8,514	6,929	36,630	+	+
3	741	779	574	835	28,657	−	−
4	978	694	14,845	10,507	45,495	−	+
5	549	733	929	728	45,079	−	−
6	28,080	13,351	709	1,012	35,389	+	−
7	53,219	35,456	720	576	38,224	+	−
8	633	930	10,103	6,623	29,487	−	+
9	1,064	857	24,063	8,256	33,147	−	+
10	1,040	841	924	1,069	13,026	−	−

* Splenic stimulator cells were obtained from mice that were injected with 200 μl of goat anti-mouse IgD antiserum 24 h before mice were killed. Cells were treated with MMC and 3×10^5 cells were added to each culture.

‡ 10^4 cloned T cells were cultured with the stimulator cells.

§ Anti-I-A^k clone (18).

‖ Arithmetic mean of [^{3}H]TdR incorporation of triplicate cultures. Underlined numbers are those that are significantly greater ($p < 0.05$) than responses to syngeneic stimulators (except responses of clone BC3C4, which are compared to incorporation of clone alone plus incorporation of stimulators alone).

and others stimulated only Mls^c-specific clones (Nos. 4, 8, and 9). Out of the total of 37 progeny tested, 16 exhibited this dissociation in the ability to stimulate only one but not the other set of Mls-specific clones. Thus, the Mls^a or Mls^a-like determinants and the Mls^c or Mls^c-like determinants expressed by CBA/J appear to be encoded by distinct and unlinked genes.

Discussion

Recently, monospecific T cell clones have proven to be powerful tools in the identification of determinants recognized by T lymphocytes that may not be detectable by serological means (23). For the analysis of Mls, under circumstances in which well-established antibodies specific for Mls gene products are not available, it appeared particularly appropriate to approach the analysis of the Mls system through the generation of cloned T cells that have specificities for different Mls gene products. In a previous communication (18), we have reported the generation and identification of Mls^a-specific clones derived from B10.BR (H-2^k, Mls^b) anti-AKR/J (H-2^k, Mls^a), and Mls^c-specific clones from B10.BR anti-

C3H/HeJ (H-2^k, Mls^c) in vitro proliferative cultures. Using these two different sets of Mls-specific clones, it was shown that Mls^a-reactive clones responded only to Mls^a stimulators but not to Mls^c stimulators, and that Mls^c-reactive clones responded only to Mls^c but not to Mls^a stimulators. None of these clones showed reactivities to both Mls^a and Mls^c stimulators. This reciprocal pattern of specificities of two sets of clones suggested that two Mls determinants, a and c, are antigenically different, and that the Mls system is therefore polymorphic. However, it was observed that all of the Mls^a-reactive clones responded to CBA/J (Mls^d) stimulators, consistent with previous reports by several investigators (15, 16). In addition, it was observed that all of the Mls^c-specific clones also responded to CBA/J. This unique crossreactivity to CBA/J stimulators by these two sets of different Mls-specific clones raised a question concerning the stimulatory determinants expressed by CBA/J splenocytes and the relationship among Mls^a, Mls^c, and Mls^d antigens.

The experiments described in this report were therefore designed to evaluate the nature of putative Mls^d determinants on CBA/J stimulators and their relationship to Mls^a and Mls^c. For this purpose, cloned T cells were positively selected for reactivity to non-MHC determinants on CBA/J (H-2^k, Mls^d) stimulators. All clones responded to the original stimulator, CBA/J. One group of clones was also stimulated by C3H/HeJ (H-2^k, Mls^c) cells while all the other clones were crossreactive to AKR/J (H-2^k, Mls^a). No clone was reactive to both AKR/J and C3H/HeJ stimulators. Since these strains express differences in many non-H-2 genes other than Mls, attempts were made to carefully define their specificities. Although the original definition of Mls-specific responses is based on the response of unprimed T cells to MHC-identical stimulators, the gene encoding Mls^a determinants has been mapped using B × D RI strains (6). Therefore, the specificity of AKR/J-reactive clones for Mls^a (or a closely linked gene) was confirmed by the pattern of responses to 20 B × D RI strains (Table III). In contrast, unlike Mls^a, formal mapping studies of Mls^c have not yet been done. Therefore, the identification of determinants recognized by C3H/HeJ-reactive cloned T cells was established by the pattern of responses to stimulators derived from congenic strains (Table IV), RI strains (Table V), and backcross mice (Fig. 2). Two C3H/HeJ-reactive B10.BR anti-CBA/J clones gave responses that were in all instances concordant with those of unprimed T cells and that therefore confirmed the Mls^c specificities of these clones. The absence of crossreactivity in the determinants recognized by these anti-Mls^a–like and anti-Mls^c–like CBA/J-reactive clones is also consistent with the previously reported characterization of Mls^a- or Mls^c-specific clones that were originally selected by Mls^a (AKR/J) or Mls^c (C3H/HeJ) stimulators (18). The ability of $Mls^{a/c}$ F_1 cells to stimulate all of the CBA/J-specific T cell clones (Table VI) strongly suggests that Mls^a and Mls^c determinants are coexpressed on CBA/J cells.

Since bulk lines of B10.BR T cells stimulated with CBA/J stimulators responded crossreactively to AKR/J and C3H/HeJ stimulators (Fig. 1), it seemed likely that these responses would be representative of normal Mls^d-reactive B10.BR T cells, and that the reactivities of cloned T cells derived from these lines would be Mls^d-specific. However, one might still argue that because Mls-specific responses are determined in primary MLR, the responses of these clones

to CBA/J stimulators might not be Mls^d-responses but rather responses to minor antigens on CBA/J cells that are only detectable by hyperstimulated cloned T cells. One such determinant has been reported by Opalka and Kolsch as Lsd (24). However, the results of primary MLR among H-2-compatible, Mls-disparate strains (Table VII) strongly supported the conclusion that determinants including Mls^a and Mls^c are also the Mls^d determinants detected by unprimed T cells. It was observed that both AKR/J (Mls^a) and C3H/HeJ (Mls^c) T cells generated primary proliferative responses to CBA/J (Mls^d) stimulators. In contrast, CBA/J T cells were unresponsive to both AKR/J and C3H/HeJ, consistent with the findings recently reported by Ryan et al. (22; Ryan J. J., J. J. Mond, and F. D. Finkelman, manuscript submitted for publication) and suggesting that genetic self-tolerance in CBA/J T cells included tolerance to both self Mls^a and Mls^c determinants, and that Mls^d therefore included the sum of Mls^a and Mls^c determinants. Although several other investigators have also observed this unidirectional stimulation of AKR/J T cells by CBA/J cells (11, 25), Molnar-Kimber and Sprent (14) failed to detect responses of AKR/J T cells to irradiated CBA/J stimulators, leading to the conclusion that Mls^a and Mls^d were antigenically indistinguishable. Under their experimental conditions, these investigators also failed to obtain anti-Mls^c responses of AKR/J T cells to C3H/HeJ stimulators. The subsequent demonstration by Webb et al. (26) that Mls^a-specific stimulation is very radiosensitive may be relevant to these findings. This radiosensitivity appears to apply to Mls^c-specific stimulation as well, since primary Mls^c-specific responses of B10.BR or AKR/J responder T cells to C3H/HeJ or CBA/J stimulators, respectively, were easily observed with MMC-treated stimulators (even in the absence of anti-IgD treatment), but were difficult to detect using irradiated stimulators (18; Ryan, J., manuscript submitted for publication). A different pattern of results was reported by Click et al. (11), who observed a bidirectional stimulation between CBA/J and C3H/HeJ splenocytes, whereas CBA/J T cells could not be stimulated by AKR/J. Although the reason for this discrepancy from the present findings is not certain, it is possible that the CBA/J anti-C3H/HeJ response reported by Click et al. (11) is due to back-reactions (27) of T cells contained in the unseparated C3H/HeJ splenocytes that were used as stimulators in those studies against Mls^d-bearing cells in the unfractionated CBA/J responders.

If Mls^d determinants consist of Mls^a determinants plus Mls^c determinants, the question remains whether unique Mls^d determinants exist in addition to those expressed by Mls^a and Mls^c strains. An attempt to answer this question was made by testing the primary MLR responses between (AKR/J × C3H/HeJ)F_1 ($Mls^{a/c}$) T cells and CBA/J stimulators (Table VII). No clearcut responses of (AKR/J × C3H/HeJ)F_1 T cells to CBA/J stimulators were observed. Furthermore, the fact that all B10.BR anti-CBA/J clones analyzed have a second reactivity to either Mls^a or Mls^c determinants, as well as an original reactivity to CBA/J stimulators, and the fact that none of them are uniquely Mls^d-specific, is consistent with the postulate that Mls^d may not be an independent Mls type.

The relationship of Mls^d to Mls^a and Mls^c was further pursued by genetic analysis. To distinguish whether theMls^a- and Mls^c-like determinants on CBA/J cells were expressed on the product of a single gene (Mls^d) or, alternatively, were

the products of separate genes, a segregation analysis was carried out with progeny of the (CBA/J × B10.BR)F_1 × B10.BR backcross. While some progeny expressed either both (Mls^a-like and Mls^c-like) or neither of the CBA/J Mls determinants, nearly half of the progeny tested (16 of 37) expressed only one and not the other of these determinants. Thus, the Mls^a-like and Mls^c-like products expressed by CBA/J appear to be encoded by distinct and unlinked genes. Perhaps the most straightforward, although not the only, interpretation of the findings presented here is that Mls^d is not an independent genotype and that Mls^d mice actually concurrently express the products of the nonallelic Mls^a and Mls^c genes. In fact, preliminary findings using segregation analysis of (AKR/J × C3H/HeJ)F_1 × B10.BR progeny indicate that the Mls^a and Mls^c genes defined in these strains are indeed non-allelic and unlinked (Abe, R., unpublished observations).

Based on all our experimental results, we would therefore like to postulate a revised and novel interpretation of the Mls system: (*a*) Two different (and in this sense polymorphic) determinants can cause primary T cell proliferation, those designated as Mls^a and Mls^c. The stimulatory capacity of the Mls^b product is not yet firmly established; (*b*) the originally proposed Mls^d phenotype is not an independent genotype but is composed of Mls^a and Mls^c gene products. Anti-Mls^d T cell responses are the sum of anti-Mls^a and anti-Mls^c responses; (*c*) An important corollary of this hypothesis is that Mls^a and Mls^c are not allelic. To date, formal mapping studies of Mls^c and clear evidence indicating the allelism of Mls^a and Mls^c have not been reported. Therefore, we would like to propose that Mls-specific responses are not controlled by a single gene, but that at least two independent genes are involved, one of which is the original Mls gene on chromosome 1 encoding Mls determinants on the lymphoid cells of Mls^a- (and Mls^d-) type mice, and the other of which has not yet been mapped and which encodes determinants expressed on Mls^c- (and Mls^d-) type mice. These two independent Mls systems appear to differ from one another in both quantitative and qualitative aspects. The precursor frequency of Mls^c-reactive T cells may be significantly less than that of Mls^a-reactive T cells, as reflected in the magnitudes of responses by unprimed B10.BR T cells to AKR/J (Mls^a) vs. C3H/HeJ (Mls^c) (Table VII) and the greater response of a B10.BR anti-CBA/J line to AKR/J vs. C3H/HeJ. In addition, the high degree of crossreactivity of alloreactive and antigen-specific cloned T cell populations to Mls^a (9, 10, 28) has not been seen to Mls^c (Abe, R., unpublished data). The influence of MHC gene products on Mls^a- and Mls^c-specific responses is also different. While Mls^a gene products can be presented in association with the majority of H-2-haplotypes (with the frequent exception of $H\text{-}2^q$ [28]), it has been reported that primary (21) and secondary (29) Mls^c-specific response are highly H-2 haplotype dependent. In fact, based on the different amplitudes and kinetics of T cell responses to Mls^a and Mls^c, Molnar-Kimber and Sprent (30) have previously suggested the possibility of nonallelism of Mls^a and Mls^c.

Although the biological significance of the Mls system is still obscure, the findings discussed here suggest that the noncrossreactive Mls^a and Mls^c determinants may represent the products of independent nonallelic genes. There would thus be no allelic polymorphism yet demonstrated within either the Mls^a

or the Mls^c system, with the exception of a possibly null or nonstimulatory allele at each of these putative loci. The Mls^a locus on chromosome 1 may therefore encode a strongly stimulatory nonpolymorphic determinant akin to the lectin-like determinant proposed by Webb et al. (15) or may influence cell interaction structures as proposed by Janeway et al. (31). Knowledge about the other genetic system, Mls^c, is very limited. The finding of clear MHC restriction in recognition of Mls^c suggests a greater similarity of this determinant(s) to conventional antigens, such as cell surface minor histocompatibility antigens, but the apparently high precursor frequency of Mls^c-reactive T cells in unprimed populations remains unexplained.

Summary

Only two sets of antigenic determinants are recognized by T lymphocytes at uniquely high precursor frequencies: those encoded by the MHC and those encoded by Mls. The structural as well as functional characteristics of MHC products have been extensively analyzed. In contrast, little information concerning the nature of Mls genes or their products is available. Although it was originally described (5, 6) that the Mls locus on chromosome 1 is composed of four alleles that encode polymorphic cell surface structures, the issues of polymorphism and allelism in the Mls system have been controversial for some time. In the present study, T cell clones were generated by continuous stimulation of B10.BR ($H\text{-}2^k$, Mls^b) T cells by CBA/J ($H\text{-}2^k$, Mls^d) stimulators and they were used to analyze the relationship of putative Mls^a, Mls^c, and Mls^d determinants. All clones proliferated in response to determinants expressed by CBA/J stimulators. In addition, each of these clones exhibited a second reactivity to either AKR/J ($H\text{-}2^k$, Mls^a) or C3H/HeJ ($H\text{-}2^k$, Mls^c) stimulators. No clone responded to both AKR/J and C3H/HeJ. These second specificities were defined to be for Mls^a or Mls^c determinants, respectively, by the response patterns of clones and unprimed T cells to stimulators derived from congenic strains, recombinant inbred (RI) strains, and backcross mice. Moreover, a segregation analysis of the (CBA/J × B10.BR)F_1 × B10.BR backcross indicated that the Mls^a-like and Mls^c-like determinants expressed on CBA/J (Mls^d) cells are in fact encoded by nonallelic, unlinked genes. These findings suggest a new concept of the polymorphism and genetics of the Mls system. It is proposed that two distinct and nonallelic gene products express, respectively, the noncrossreacting Mls^a and Mls^c determinants, and that the Mls^d phenotype does not represent an independent genotype but rather reflects the concurrent expression of Mls^a and Mls^c. The Mls system, therefore, consists of at least two systems that are distinct both genetically and antigenically, and that may be of different biologic or physiologic significance as well.

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