Immunogenetics 10: 481–497, 1980

Immuno-genetics

T Lymphocytes Responding to Mls-Locus Antigens Are Lyt-1$^+$, 2$^-$ and *I-A* Restricted

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Abstract. We have investigated primary and secondary responses of mouse splenic T cells to strong mixed lymphocyte stimulating antigens controlled by the *Mls* locus using MHC-identical mixtures of cells. Our studies show that strong primary *Mls*-locus specific responses involve recognition of self I-A antigens, since BUdR and light suicide or F1 into parent radiation bone-marrow chimeras both demonstrate a preference of unprimed F1 T cells to respond to Mls-locus antigens associated with one parent's MHC antigens. Furthermore, conventional anti-I-A antisera and monoclonal anti-I-A antibody both inhibit *Mls*-locus responses in an MHC-specific manner. Finally, as is typical of T cells responding to I-A antigens or to nominal antigens associated with self I-A, *Mls*-locus responses are mediated by Lyt-1$^+$, 2$^-$ cells. One striking finding in these studies was the very high frequency of cells capable of responding to Mls-locus antigens, the highest being 1/300 splenic T cells. This plus evidence for recruitment during primary *Mls*-locus responses may account for reports of a lack of *I-A* restriction in secondary anti-*Mls* locus responses to strong Mls-locus antigens, a finding with which we concur. The possibility that these secondary responses between noncongenic strains of mice may be directed at other genetic loci is also discussed. These experiments leave open the question of the biological role of the *Mls*-locus and of the very large number of T cells reactive to it.

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Abbreviations used in this paper: MHC = Major histocompatibility complex; Mlg = Mouse immunoglobulin; MLC = Mixed lymphocyte culture; TCGF = T-cell growth factor.

0093-7711/80/0010/481/$ 3.40

Introduction

Vigorous primary in vitro T-cell proliferative responses are stimulated by cells from animals differing from the T-cell donor at the major histocompatibility complex (MHC) (Bach et al. 1972). The strength of these responses appears to reflect a very high frequency of precursor T cells specific for foreign (i. e., nonself) MHC antigens (Wilson et al. 1968, Binz and Wigzell 1977, Ryser and McDonald 1979). The only other antigens known to stimulate strong primary in vitro proliferative responses in unfractionated T cells are those encoded by the *Mls* locus in the mouse (Festenstein 1973). While the molecular nature of Mls-locus antigens is not known, it would appear that these antigens are present on B cells and/or macrophages. They can be detected at present only by stimulation of T-cell proliferation in mixed lymphocyte culture (MLC). These antigens have been mapped to chromosome 1 in the mouse (Festenstein et al. 1977). Unlike responses directed at MHC antigens, T cells responding to Mls-locus antigens do not express cytotoxic potential (Peck et al. 1977 a). *Mls*-locus disparate T cells will stimulate antibody production by B cells in an antigen-nonspecific fashion, but *Mls*-disparate T and B cells can also cooperate in an antigen-specific fashion (Janeway 1976). That is, restriction of T-B interaction by Mls-locus antigens has not been observed.

From these findings, it is clear that the *Mls* locus behaves similarly to but not identically with the *I-A* subregion of the MHC. This suggested to us that Mls-locus antigens might be recognized in association with I-A antigens. In an initial study of the responses of Mls^b T cells to Mls^c stimulator cells, it was shown that MHC-identical, Mls^b anti-Mls^c T blasts recognized not only the Mls^c antigen, but also an I-region product of the stimulator cell (Peck et al. 1977 b). The present studies were initiated to define further T-cell responses to Mls-locus antigens. In particular, we wished to determine whether primary responses to Mls-locus antigens involved recognition of I-region gene products, which gene products in the *I* region were involved, and which subpopulation of T cells was responding.

From these studies, we can conclude that T cells responding to Mls-locus antigens are primarily Lyt-1^+, 23^- cells and that the response involves the recognition of I-A-subregion gene products. We have demonstrated that this restriction by I-A-region products is present in the primary response. One surprising and as yet unexplained finding is that the frequency of precursor T cells specific for certain Mls-locus antigens is as high as or higher than the frequency of T cells recognizing allogeneic MHC-gene products. We conclude that the response to Mls-locus products represents a model system for the study of Ly1 T-cell responses to antigens recognized in association with I-A-subregion gene products. These Ly1 T cells are precommitted to recognizing Mls-locus antigen associated with particular I-A antigens. We have also found an MHC-unrestricted component to this response, seen when using primed T cells or T-cell lines, suggesting genetic complexity in non-MHC, T-cell stimulating antigens.

Materials and Methods

Mice. BALB/cByJ, C57BL/6J, CBA/J, C3H/HeJ, DBA/2J, (AKR × DBA/2)F_1 (AKD2F1), SJL/J, AKR/J, CBA/CaJ, and B10.D2/$_0$Sn mice were all obtained from the Jackson Memorial Laboratories.

B10.BR, (BALB/c × C3H/HeJ)F_1, (BALB/c × B10.BR)F_1 and BAB.14 mice were bred in our own colonies at Yale. Mice were used at 8 weeks to 6 months of age and were all of one sex in any given experiment.

Antisera. Rabbit anti-mouse immunoglobulin (Rabbit anti-MIg) was prepared by injecting rabbits with DEAE-purified MIg in adjuvant i. p., followed by intravenous boosting with alum-precipitated MIg. Anti-Thy-1.2 was prepared as previously described (Janeway 1975). Anti-Lyt-1.2 and anti-Lyt-2.2 were prepared as previously described and were the kind gift of Dr. Fung-Win Shen (Janeway et al. 1980). A.TH anti-A.TL sera were prepared and donated by Dr. Donal B. Murphy. Monoclonal anti-K^k and anti-I-A^k reagents were hybridoma products donated by Drs. Herzenberg, Herzenberg and Oi of Stanford University. Anti-K^k and anti-D^d used to characterize the chimeric mice were donated by Dr. Donal Murphy.

Fluorescent staining. Direct and indirect staining for Thy-1.2, Lyt-1.2, Lyt-2.2, surface MIg, K^k and D^d products were carried out as previously described (Janeway et al. 1980).

Mixed Lymphocyte culture. Two types of culture were carried out. Analytical cultures were carried out in EHAA medium (Click et al. 1972) supplemented with 5% heat-inactivated fetal calf serum in flat bottom microtiter trays (Falcon, 3040). Varying numbers of Ig-anti-Ig column (Wigzell 1976) purified spleen T cells were incubated with 5×10^5 mitomycin C treated stimulator spleen cells, pulsed for 3 h with 1 μCi ^{3}H-thymidine (^{3}H-TdR, 50–60 Ci/m mole) New England Nuclear), harvested on a MASH-II harvester (Microbiological Associates, Bethesda, Maryland) and counted in a liquid scintillation counter (Beckman Instruments). Results are expressed as the mean of triplicate counts per minute, with the background given by syngeneic cells subtracted (CPM, E-C). Titration curves of response versus number of responder T cells added per well were plotted on log-log paper, and the number of interacting responding cells determined from the slope of the dose-response curve (Mosier and Coppleson 1968). Bulk MLCs were prepared in an identical fashion except the cultures were carried out in Falcon 3013 flasks in 12 ml medium containing 0.5% fresh autologous normal mouse serum instead of fetal calf serum.

Isolation of T blasts from MLC cultures. T blasts were isolated from bulk cultures either by unit gravity sedimentation in a linear gradient of fetal calf serum (Peck et al. 1977 a) or by centrifugation on a stepwise gradient of Percoll (Pharmacia Fine Chemicals, Piscataway, New Jersey), according to a method given to us by Dr. James Kurnick (personal communication). When staining on blasts is reported, only those cells greater than twice the diameter of normal lymphocytes were counted.

MLC T-cell lines. Isolated T-cell blasts from primary bulk MLC were sustained in culture by twice-weekly changes of two day Con A spleen cell supernatant. They were rechallenged with stimulator cells plus Con A supernatant (T-cell growth factor, or TCGF) every 10 days. Before testing, cells were washed three times and counted.

Blocking of MLC with antisera. Either monoclonal antibody, or purified conventional antibodies were added to MLC cultures prior to the addition of the responder T cells, so that the antibodies could react with the stimulator cells. In using conventional anti-MHC antisera, 50% saturated ammonium sulfate precipitates were further purified by passage over sephadex-G-200 columns, dialysed, concentrated by ultrafiltration and added to culture. Adsorptions of antisera were performed with whole spleen cells as noted in the text.

Preparation of radiation chimeras. A variety of techniques were tried. The most successful chimeras were produced using bone-marrow cells from donors treated with 0.2 ml rabbit anti-mouse thymocyte antiserum (Microbiological Associates, Lot # 3822) two days prior to transfer (Kappler and Marrack 1978), and recipients X-irradiated with 925R 1 day prior to transfer. The bone-marrow cells were further treated with AKR-anti-C3H thymocyte serum and guinea pig complement as previously described (Janeway 1975), and 5×10^6 viable cells injected i. v. The mice were kept for 1 to 9 months before testing.

5-Bromodeoxyuridine and light suicide. The previously employed techniques were also used in these experiments (Janeway et al. 1978). Recovered cells were restimulated in MLC culture as described above and pulsed daily; only the maximum response is reported.

Results

The purpose of the present experiments was to characterize the T cells responding to Mls-locus antigens. In particular, we wished to determine whether such cells responded to the *Mls* locus per se, or whether recognition of Mls-locus antigens also involved recognition of self MHC antigens. Since we wished to determine whether the MHC restriction existed prior to contact with the antigen, we examined the primary response to Mls-locus antigens. For this reason, we selected responses of mice of *Mls*-locus types Mls^b or Mls^c against stimulators from mice of *Mls*-locus types Mls^a or Mls^d.

Responding cells in primary Mls-locus MLC reactions are Lyt-1$^+$, 2$^-$ Tcells. Spleen cells, splenic T cells, or Lyt-1$^+$, 2$^-$ (Ly1) splenic T cells were prepared by fractionation on Ig-anti-Ig columns and tested for reactivity in MLC responses. A representative experiment (of three) is shown in Table 1. B10.D2 (Mls^b) cells react well with MHC-identical DBA/2J (Mls^a) stimulators, as well as with H-2^k and H-2^s stimulators. The response to DBA/2 is progressively enriched in T cells and Ly1 cells. The cell-surface Lyt antigen phenotype of each of the cell populations as determined by cell-surface immunofluorescence is given immediately below. It is clear that purified Ly1 T cells respond vigorously to strong Mls-locus antigens. The decreased response of these cells to *H-2* was not seen in all experiments.

That the blast cells generated in *Mls*-locus responses are also predominantly Ly1 cells is shown in the experiments presented in Table 2. Here, T cells were

Table 1. Purified Ly1 T cells respond to Mls^a and *H-2* differences in MLC

			Responding B10.D2 population*		
Stimulator cells	*H-2*	*Mls*	Spleen	Spleen T†	Spleen Ly1 T†
B10.D2	*d*	*b*	(735)	(1035)	(655)
B10.BR	*k*	*b*	5167‡	44638	12184
A.SW	*s*	*c*	22009	63165	24805
DBA/2	*d*	*a*	17083	68727	122853
Responding population stained with			Percent staining cells		
Rabbit anti-Mouse Ig			55%	0%	0%
Anti-Thy-1.2‖			37%	85%	74%
Anti-Lyt-1.2§			—	61%	69%
Anti-Lyt-2.2¶			—	24%	2%

* 2×10^5 responding cells added to 4×10^5 mitomycin C-treated spleen cells. ^{3}H thymidine uptake measured 4 days later.

† Spleen cells passed over Ig-anti-Ig columns to produce spleen T. Spleen T incubated 30′ at room temperature with 1 : 10 anti-Lyt-2.2, washed twice and passed over Ig-anti-Ig columns to yield Ly1 T.

‡ Mean cpm of culture minus control cpm with syngeneic stimulator cells.

‖ Direct fluorescence with FITC-conjugated AKR anti-C3H thymocyte antiserum.

§ Indirect fluorescence using C3H/An anti-CE (anti-Lyt-1.2) followed by FITC-rabbit anti-MIg.

¶ Indirect fluorescence using C3H/B anti-ERLD (anti-Lyt-2.2) followed by FITC-rabbit anti-MIg.

Table 2. Lyt-antigen phenotype of T-cell blasts isolated from MLC cultures

		Differences responder-stimulator*				Percent blasts staining for †		
Responding T cell	Stimulator	*H-2K*	*I-A*	*H-2D*	*Mls*	Thy 1.2	Lyt-1.2	Lyt-2.2
(C3H × BALB/c)F_1	DBA/2	−	−	−	+	85%	80%	33%
(C3H × BALB/c)F_1	DBA/2	−	−	−	+	94%	72%	20%
(C3H × BALB/c)F_1	DBA/2	−	−	−	+	96%	78%	13%
(C3H × BALB/c)F_1	AKR/J	−	−	−	+	91%	81%	12%
(C3H × BALB/c)F_1	AKR/J	−	−	−	+	91%	79%	19%
(C3H × BALB/c)F_1	AKR/J	−	−	−	+	97%	80%	20%
(C3H × BALB/c)F_1	BALB.B	+	+	+	−	93%	56%	65%
BALB/c	C57BL/6	+	+	+	−	96%	66%	49%
BALB/c	A/J	+	+	−	+	93%	68%	45%
BALB/c	A/J	+	+	−	+	99%	60.5%	50.5%
A.TH	A.TL	−	+	−	−	95%	76%	17%

* + = difference at locus between responder and stimulator; − = no difference at locus between responder and stimulator.

† Cells were cultured 3–5 days in EHAA with 0.5% normal mouse serum. Blasts were isolated by either velocity sedimentation for 3 h in dilute fetal calf serum or by centrifugation on Percoll gradients. Staining on blasts only was determined. See Table 1 for stains used.

cultured with stimulators for 3–5 days in medium containing normal mouse serum. The blasts were then isolated and their cell-surface antigen phenotype determined by immunofluorescence. About 80 percent of T blasts from *Mls*-locus MLC responses are Ly1 cells and only 20 percent are Ly2 cells, while T blasts from responses involving all or part of the MHC consist of 40 percent Ly1 cells, 25 percent Ly2 cells and 25 percent Ly12 cells. Finally, responses to isolated *I*-region MHC differences consist of 76 percent Ly1 cells and 17 percent Ly2 cells. Thus, responses to Mls-locus antigens strongly resemble reactions to isolated *I*-region differences in that they are mediated predominantly or exclusively by Lyt-$1^+, 2^-$ T cells. Unlike reactions involving K- and/or D-region antigens, responses to Mls-locus antigens do not involve sizable numbers of Lyt-2^+ cells (20 percent versus 50 percent).

Anti-Ia antibodies specifically inhibit primary Mls-locus MLC responses. Responses to Mls-locus antigens resemble responses to isolated *I*-region differences in generating strong proliferative respones in Ly1 T cells, which are known to respond selectively to self or allogeneic *I-A* (Cantor and Boyse 1977). For this reason, it seemed likely that *Mls*-locus responses would involve recognition of *I*-region encoded structures. We had previously shown this in secondary *Mls*-locus MLC reactions to the weak Mls^c antigen. In the present experiments, we wished to determine if primary *Mls*-locus responses to strong Mls-locus antigens also involve *I*-region recognition. In order to do this, T cells from $H\text{-}2^k \times H\text{-}2^d$, Mls^b or $Mls^b \times Mls^c$ F_1 mice were reacted with stimulator spleen cells from $H\text{-}2^k$ or $H\text{-}2^d$ mice, and the effect of anti-Ia antisera on the response determined. The results obtained using various fractions of an A.TH anti-A.TL (anti-Ia^k) antiserum following absorption with BALB/c ($H\text{-}2^d$, Mls^b) spleen cells are shown in Table 3.

Table 3. Inhibition of the response of T cells to *Mls* locus with anti-Ia antibody

Stimulators (2000 R)	*H-2*	*Mls*	Responders: (C3H × BALB/c)F_1 spleen T cells*			
			Response in PBS (CPM, E–C)	Residual response in anti-$Ia^{k\dagger}$ (as percent of control response)		
				IgM	IgG	IgG 1 : 5
(C3H × BALB/F_1	*kxd*	*bxc*	(1 007)	(677)	(1932)	(1120)
AKR	*k*	*a*	43 332	23	8	37
C57BL/6	*b*	*b*	78 229	247	274	141
CBA/J	*k*	*d*	159 964	15	13	54
DBA/2J	*d*	*a*	187 661	93	111	105
(AKR × DBA/2)F_1	*kxd*	*a*	183 345	96	40	127

* MLC culture: Spleen cells were purified over Ig-anti-Ig columns, and cultured at 1.5×10^5 per well in flat bottom plates with 5×10^5 irradiated spleen cells as stimulators. Stimulators were incubated 1 h with anti-Ia before the addition of responders. Medium was EHAA with 5% heat-inactivated fetal calf serum.

† Antiserum: A.TH anti-A.TL, ammonium sulfate precipitate passed on Sephadex G-200, 19S and 7S peaks taken, dialysed v. PBS, concentrated by Amicon, sterile filtered, and absorbed with BALB/c spleen cells. 1 ml serum gave rise to 5 ml of each peak, and 20 µl of each peak was used in 200 µl of culture fluid. Absorption was with cells from one spleen per 0.3 ml of each peak.

This antiserum inhibits responses to Mls-locus antigens on H-2^k but not on H-2^d stimulator cells; it is not inhibitory of responses to H-2^b either. Most important, partial or no inhibition of responses to H-2^k × H-2^d, Mls^a stimulator cells was seen, supporting the notion that the antiserum is blocking T-cell recognition of the antigen rather than simply killing the stimulator cell.

Having obtained this result, we next examined the subregion(s) recognized in this response by absorbing the antiserum on various B10 congenic and recombinant strains as shown in Table 4. It is clear that the majority of the activity is directed at products of the *I-A*^{k} subregion (Aα^k and/or Aβ^k), since cells from strain B10.A(4R) will absorb much of the activity, while cells from strain B10 will not. These two strains differ only in the *I-A* subregion within the *I* region. A role for other I-subregion antigens can not be ruled out.

Finally, similar studies were carried out using anti-K^k and anti-I-A^k monoclonal antibodies. The results of five similar experiments are presented as normalized percent residual responses in Table 5. It is clear that anti-K^k monoclonal antibody has no significant effect on these response, whereas anti-I-A^k consistently blocks responses to AKR/J and CBA/J (H-2^k) stimulators. Responses to C57BL/6J (H-2^b) and DBA/2J (H-2^d) are not significantly reduced. Finally, responses to (AKR × DBA/2J)F_1 stimulators are intermediate between those to the parental stimulator.

We have consistently observed partial inhibition of the response to (AKR × DBA/2)F_1 stimulators with anti-I-A^k antisera and monoclonal antibodies (Tables 3–5). We attribute this partial blocking to the presence of two sets of responding T cells derived from the F_1 mice: one set directed at Mls^a associated with *I-A*^{k} and the other directed at Mls^a associated with *I-A*^{d}. The response of the former

Table 4. Blocking of *Mls*-locus mixed lymphocyte reactions with antibodies specific for *I-A*

Responders: (B10.BR × BALB/c)F_1 spleen T cells						
Stimulating cells	*H-2*	*Mls*	Control response*	Percent residual thymidine incorporation in A.TH anti-A.TL absorbed with:†		
				BALB/c + B10	BALB/c + B10.BR	BALB/c + 4R
B10.BR × BALB/c)F_1	*kxd*	*b*	(2144)	(1731)	(1599)	(1710)
AKR	*k*	*a*	21 564	16	80	55
C57BL/6	*b*	*b*	68 578	74	92	78
CBA/J	*k*	*d*	13 832	12	94	69
DBA/2J	*d*	*a*	15 740	104	84	175
(AKR × DBA/2)F_1	*kxd*	*a*	31 590	42	82	108

* See Table IV, Footnote*.

† Absorptions were carried out with 3×10^8 spleen cells per 400 µl of antibody, which is equivalent to approximately 40 µl of antiserum. See Table IV, Footnote†.

set to the F_1 stimulator would be blocked by anti-I-A^k, while that of the latter would not, leading to the observed result.

Evidence for two distinct populations of T cells responding to Mls-locus antigens in H-2 heterozygous mice. In order to determine if the H-2^k × H-2^d, Mls^b × Mls^c T cells contained two distinct populations of T cells reacting to Mls^a antigens, one in association with *I-*A^d and the other in association with *I-*A^k, as suggested by the data in the previous section, the following experiments were performed. F_1 T cells were mixed with stimulator cells, and the dividing cells eliminated by means of 5-bromodeoxyuridine and light treatment. The residual cells were washed and restimulated with various types of stimulator cells. The results are given in Table 6. While these results do not show complete discrimination between T cells responding to Mls^a antigens associated with one or the other parental *MHC*

Table 5. Inhibition of *Mls*-locus responses with monoclonal anti-I-A antibody

Stimulator spleen cells			Average Percent residual proliferation with*:	
Strain	*H-2*	*Mls*	10.2.16 (anti-I-A^k)	11.4.1 (anti-K^k)
AKR/J	*k*	*a*	13.7 ± 1.6	144.4 ± 4.1
C57BL/6J	*b*	*b*	160.9 ± 5.0	158.2 ± 7.7
CBA/J	*k*	*d*	20.5 ± 4.3	196.0 ± 25.2
SBA/2J	*d*	*a*	90.8 ± 3.0	124.8 ± 5.4
AKD2F_1	*kxd*	*a*	53.0 ± 5.5	132.4 ± 6.6

* Spleen T cells at 10^5/well from (BALB/c × C3H)F_1 mice were added to 5×10^5 mitomycin C treated spleen cells which had been incubated for 1 h with the monoclonal antibody before adding the responder cells. Antibody was present throughout the culture. Cultures were pulsed and harvested 4 days later, and the results calculated as the percent of the response in the absence of antibody. These normalized results from five experiments were then averaged to generate the numbers in this table, which are the mean percent residual response ± 1 s.e.

Table 6. Partial MHC restriction of 5-bromo-deoxyuridine and light suicide of *Mls*-locus response

Responders: (B10.BR × BALB/c)F_1 spleen cells suicided against (CPM, E–C)						
Stimulators	Fresh F_1 (A)	C57BL/6	DBA/2 (B)	AKR/J (C)	(A)+(B)	(A)+(C)
C57BL/6J	194417	2241	17695	34405	189428	224133
DBA/2J	142750	90078	51538	83393	60544	80841
AKR/J	67321	15030	22525	9213	33704	33257
CBA/J	128880	118285	71153	72960		

Responder spleen cells were incubated with stimulators and 5-bromo-deoxyuridine, 3×10^{-6}M added after 27 h. Cultures were lit at 69 and 92 h for 90 min. Remaining cells were recultured with fresh stimulators as shown. Plates were pulsed 3,4, and 5 days after reculture with tritiated thymidine and harvested 3 h later. Values given are peak responses in each instance, expressed as counts above control response to irradiated F_1 stimulators on that day. Underlined values are the lowest response to that stimulator. The last two columns represent responses of 1 : 1 mixes of fresh F_1 cells with suicided cells.

haplotype, they are consistent with this interpretation. The failure to achieve total elimination of responses in this system may be related to the very high precursor frequency of Mls-reactive cells in this combination (see below).

H-2-restricted responses to Mls-locus antigens in F_1 into parent radiation chimeras. Zinkernagel and many other investigators (see Zinkernagel and Doherty 1979) have employed radiation chimeras to demonstrate MHC restriction in various antigenic systems. In order to demonstrate MHC restriction in Mls-locus responses further, such chimeras were constructed using the same strain combinations already employed in the anti-Ia blocking studies and the BUdR and light experiments. We found that it was necessary to use vigorous treatment to eliminate residual donor T cells in order to obtain chimeric mice giving *H-2*-restricted responses to Mls-locus antigens, as shown in Table 7. As in the BUdR and light experiments, the degree of restriction to the *H-2* type of the parental recipient is not absolute, but it is consistent with the proposition that responses to Mls-locus antigens are MHC restricted. The degree of chimerism is very high, as determined by immunofluorescence of the splenic T cells which allows detection of as few as 1 percent of recipient cells.

T cells responding to Mls-locus antigens are present in normal mice at very high frequency. Because responses to Mls^a and Mls^d are very intense and because of our difficulty in eliminating Mls-reactive cells by BUdR and light or in donor bone-marrow cells in radiation chimeras, it seemed likely that *Mls*-locus responsive T cells would be present at a very high frequency in our responding T-cell populations. Therefore, two different techniques were employed to estimate the frequency of responding cells in these responses. Both involved limiting dilution of the responding T cells mixed with a fixed number of stimulator spleen cells.

The first technique is simply to construct log-log dose response curves of response versus T cell number per well, as shown in Figure 1 (Corley 1977). It is clear that in this system responses to Mls-locus antigens exceed those of the same T cells to two allogeneic stimulators, C57BL/6 and SJL. This is true at all cell doses and at

Table 7. T cells from F_1 parent chimeras show MHC-restricted response to Mls-locus antigens

Stimulator	*H-2*	*Mls*	Proliferative response (CPM, E–C) of spleen T cells from: Normal F_1*	kxd→d chimera†	kxd→k chimera‡
F_1	*kxd*	*b*	(3267)	(1544)	(8273)
C57BL/6J	*b*	*b*	46191	47405	69024
CBA/CaJ	*k*	*b*	2328	2673	7816
AKR/J	*k*	*a*	79116	4451	16979
B10.D2/oSn	*d*	*b*	176	1519	0
DBA/2J	*d*	*a*	140052	4492	0
Experiment 2					
F_1	*kxd*	*b*	(2664)	(7071)	(3673)
C57BL/6J	*b*	*b*	61725	52096	36602
CBA/CaJ	*k*	*b*	1641	962	2612
CBA/J	*k*	*d*	12801	0	9978
B10.D2	*d*	*b*	2849	2752	768
DBA/2	*d*	*a*	55834	16081	11323

* Spleen T cells from normal (BALB/c × C3H/HeJ)F_1 mice.

† Spleen T cells from chimera of (BALB/c × C3H/HeJ)F_1 bone marrow given to 925 R x-irradiated B10.D2/oSn recipients 8 or 16 weeks before testing. Degree of chimerism: T cells 96% donor type (K^k positive), experiment 1, 94% donor type, experiment 2.

‡ Spleen T cells from chimera of (BALB/c × C3H/HeJ)F_1 bone marrow given to 925 R x-irradiated CBA/CaJ recipients 8 or 16 weeks before testing. Degree of chimerism: T cells 97% donor type (D^d positive), experiment 1, 94% donor type, experiment 2.

all times tested, as shown in Figure 2. It can also be seen from these two experiments that the responding T-cell population behaves as though it contained synergizing cell sets, since the increase in the response with increasing responder cell number exceeds the increase in the cell number. This is seen most clearly from the slope of the log-log dose response curve. These slopes range from 1.3 to 1.7. Furthermore, the slope increases with time in the response, strongly suggesting recruitment (Fig. 2). Finally, these graphs illustrate one of the potential problems of studying MLC responses at a particular time and cell dose: namely, that at a given cell dose, a response to one stimulator may appear to be less than that to another stimulator, yet in fact the opposite is true, since at high responder cell numbers, diminshed responses to strong stimulators may occur.

Second, we have performed a fluctuation analysis of this response at limiting dilution and determined precursor frequency in the absence of enhancing supernatants by both Poisson analysis of total responding wells and by determining the number of probable responding cells per well at limiting dilution by analysis of clustering of data points. The two methods give good agreement, as shown in Table 8. As can be seen, we find a very high precursor frequency for responses to Mls^a, especially on $H\text{-}2^k$ stimulator cells. Precursor frequencies for responses to Mls^a and Mls^d are comparable to those obtained for whole MHC differences with $H\text{-}2^b$ and $H\text{-}2^s$ stimulators. Thus, the vigorous primary in vitro responses associated with Mls-locus antigens are reflected in a high frequency of T cells responsive to these antigens.

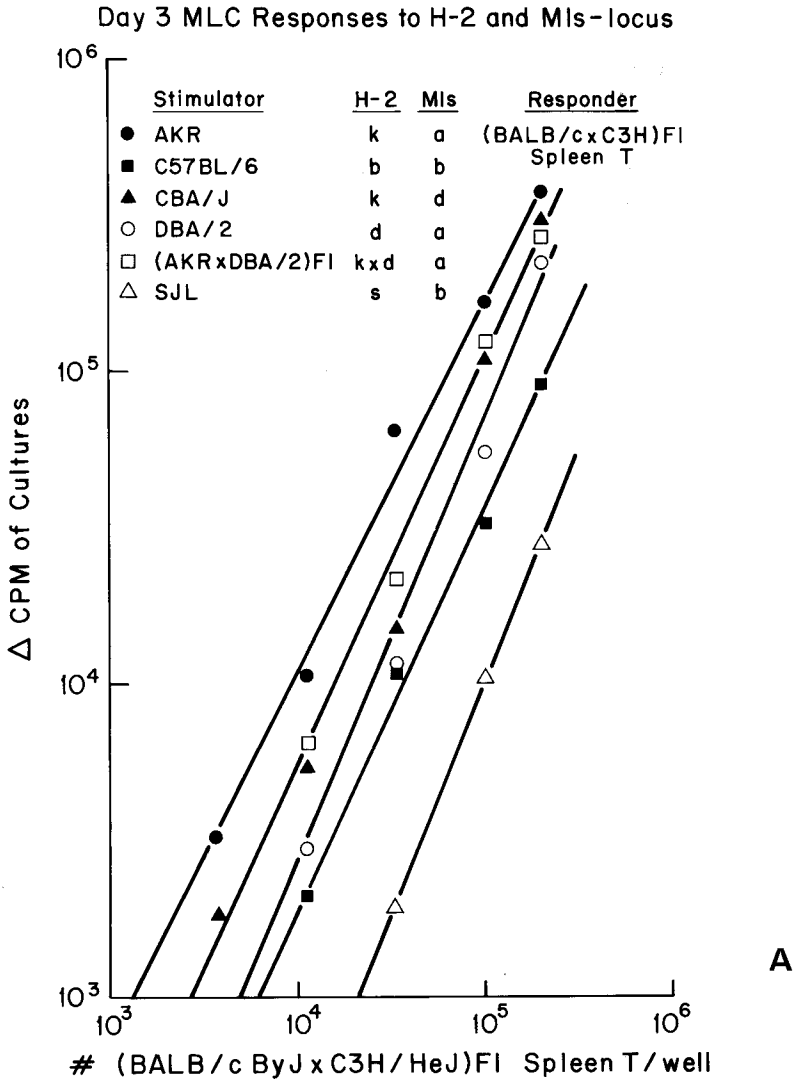
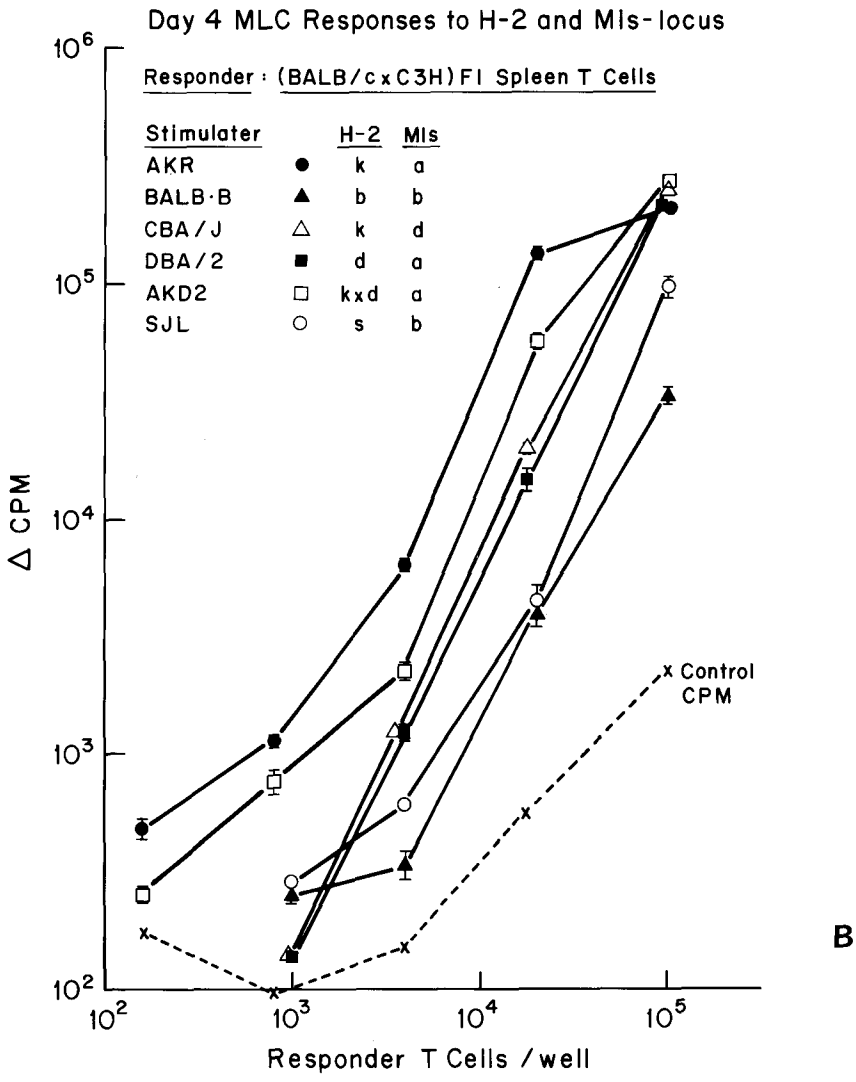


Fig. 1 A and B. Log-log plot of the day 3 (**A**) or day 4 (**B**) T-cell proliferative response to a fixed number of stimulator cells. Responder cells are (BALB/c × C3H)F_1 spleen T cells at varying numbers, added to 5 × 10^5 mitomycin C treated stimulators. Thymidine incorporation was measured after a 3 h pulse. Slopes of the dose response curves range from 1.3–1.7 in these two experiments, except at a very low cell dose, where they are approximately equal to 1.0.

Failure to demonstrate MHC-restricted responses to Mls-locus antigens using isolated specific T blasts. Other authors have used isolated T blasts to study the secondary in vitro response to Mls^a or Mls^d locus antigens and have reported that these responses are not MHC restricted (Molnar-Kimber and Sprent 1980). This seemed surprising, since similar experiments by our team (Peck et al. 1977 b) and by Delovitch and Sohn (1979) showed excellent MHC restriction in response to Mls^c



antigens and since the present experiments pointed to *I-A* restriction of Mls^a and Mls^d directed responses. Therefore, anti-Mls^a blasts were prepared using F_1 responder cells and homozygous stimulators. The secondary proliferative responses of these cells are shown in Table 9. It can be seen that there is no MHC restriction in this response, and this was true at all cell doses tested, and in several experiments. There are several possible explanations for these results, which would appear to conflict with the data presented previously in this paper, and these will be discussed below.

This result is strengthened by findings using BALB/c and BAB.14 (*H-2*^{d}) T-cell lines responsive to DBA/2, as shown in Table 10. In this instance, no responses to H-2^d or H-2^k per se are seen. However, strong responses to Mls^a or Mls^d antigens on

Table 8. Precursor frequency of responding T cells to MHC or Mls-locus antigens

Responding cells: Spleen T from (BALB/cByJ × B10.BR)F_1				
			Reciprocal of precursor frequency* analysed by:	
Stimulator	*H-2*	*Mls*	Poisson distribution†	Scatter diagram‡
AKR/J	*k*	*a*	450	240
C57BL/6J	*b*	*b*	4000	N.D.
CBA/J	*k*	*d*	700	888
DBA/2J	*d*	*a*	3000	3200
(AKR × DBA/2)F_1	*kxd*	*a*	310	343
B10.S	*s*	*b*	600	654

* Varying numbers of spleen T cells were added to 5×10^5 Mitomycin-C treated spleen cells of various types, including (BALB/cByJ × B10.BR)F_1 cells. Twenty-four wells at each dilution of cells were pulsed for 3 h with ^{3}H-TdR on day 4, harvested and counted.

† Determined from the number of responder cells/well where 63% of cultures gave responses above background.

‡ Determined by counting the number of wells giving responses expected from one, two, three, etc. precursors, multiplying the number of such wells times the numbers of precursors in the well, and dividing the total numbers of precursors found by the total number of cells cultured at that dilution.

H-2^d or H-2^k stimulator cells are found. The differences in response to the various stimulators suggest that these responding cells may be quite heterogeneous, containing both MHC-restricted and MHC-unrestricted components, as well as cells that do or do not cross-react between Mls^a and Mls^d.

Discussion

The present experiments demonstrate that the recognition of Mls-locus antigens in primary responses involves both the Mls-locus antigen and a product (products) of

Table 9. Secondary Response to Mls-locus Antigens does not show MHC Restriction

Responders are (BALB/c × C3H)F_1 spleen T					
			48 h proliferative response by 10^4 T blasts		
Stimulator	*H-2*	*Mls*	F_1-anti-AKR	F_1-anti-DBA/2	Fresh F_1†
F_1	*kxd*	*bxc*	(1669)	(2157)	(562)
AKR/J	*k*	*a*	11042	25368	135993
C57BL/6J	*b*	*b*	826	6851	3905
CBA/J	*k*	*d*	10492	44218	20786
DBA/2J	*d*	*a*	12738	33128	14861
(AKR × DBA/2)F_1	*kxd*	*a*	12594	31971	57275

* Responder cells were recovered as blasts from day 4 primary bulk MLC cultures in EHAA plus 0.5% F_1 normal mouse serum by Percoll sedimentation, washed, and cultured 24 h before restimulation. For Ly phenotype, see Table 3.

† 2×10^4 fresh F_1 T cells used for generating blasts assayed at day 4 of primary culture.

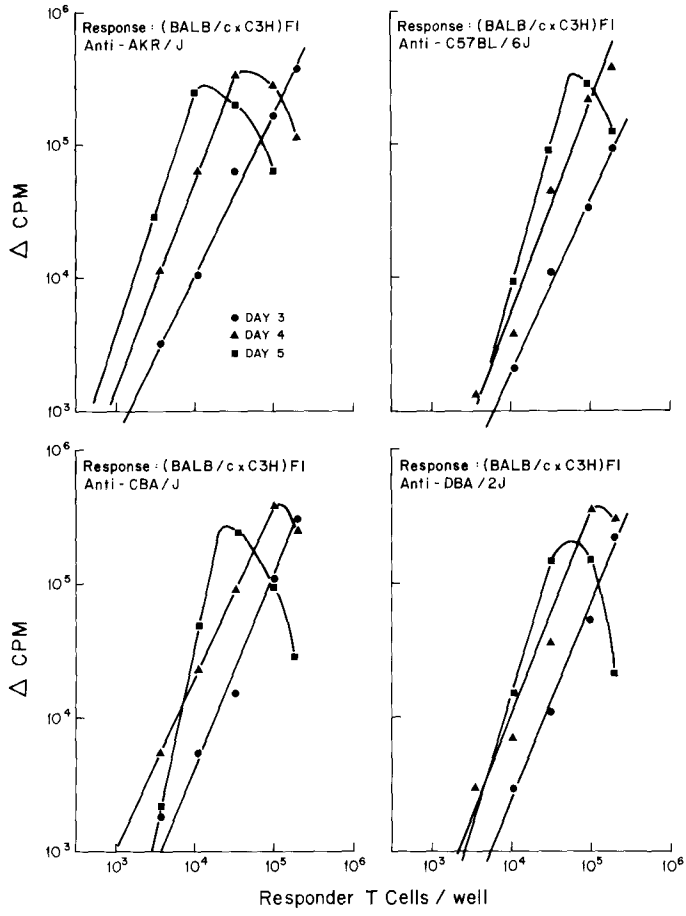


Fig. 2. Log-log plot of the response of (BALB/c × C3H)F_1 spleen T cells to a fixed number of mitomycin C treated stimulators, on days 3 (●), 4 (▲), or 5 (■). Slopes of the dose response curves range from 1.3 to 2.0.

I-A-region genes. The current evidence involves blocking of *Mls*-locus responses with anti-I-A antibodies directed at the A_α or A_β chains, but not with anti-H-2K antibodies, selective BUdR and light suicide of F_1 T cells responding to stimulator cells bearing Mls-locus antigens and MHC antigens of one of parental strains, and radiation chimeras of the type F_1 into parent. Findings of MHC-restricted secondary responses to Mls-locus antigens have been presented by several laboratories (Peck et al. 1977b, Delovitch and Sohn 1979, Kastner et al. 1977).

However, it would appear that responses to strong (Mls^a and Mls^d) Mls-locus antigens may have an MHC-independent component as well, since F_1 T cells primed in vitro with Mls^a associated with one parent's MHC respond to Mls^a or Mls^d associated with either parent's MHC antigens, a result that was not found in previous studies with Mls^b anti-Mls^c T blasts. This lack of MHC restriction may represent responses to non-Mls-locus antigens that are non-MHC restricted and

Table 10. Responses of T cells cultured 1 month with T-cell growth factor following priming with Mls-locus antigens

Stimulators	*H-2*	*Mls*	Day 2 proliferative responses (CPM,E–C) of primed cell lines*: BALB/c-anti-DBA/2	BAB.14-anti-DBA/2
BALB/c	*d*	*b*	(121)	(100)
DBA/2J	*d*	*a*	32339	30101
BALB.K	*k*	*b*	113	0
AKR/J	*k*	*a*	18539	23214
CBA/J	*k*	*d*	8547	10281
A.TH	*s/s/d*†	*c*	0	34
A.TL	*s/k/d*	*c*	129	161
TCGF	–	–	12152	11601

* BALB/cByJ or BAB.14 T cells were cultured with mitomycin C-treated DBA/2 stimulators, the blasts collected by Percoll separation and cultured in the presence of T-cell growth factors. They were stimulated with mitomycin C-treated DBA/2 spleen cells every 10 days. Forty-five days after setting up primary cultures, 10^4 viable cells were added to 5×10^5 stimulators, and cultures harvested 2 days later.

† *s/s/d* refers to alleles at the *K, I-A and D* regions of *H-2*.

are selectively detected in secondary MLCs (Peck et al. 1978), recruitment of nonspecific T cells during the primary in vitro response (Figs. 1 and 2), or a true non-MHC-restricted response to the Mls-locus antigens themselves. Further experiments using cloned Mls-reactive T cells and congenic strains differing only at the *Mls* locus will be required to resolve these points. Our data showing a very high precursor frequency of Mls-reactive T cells and recruitment in this response would tend to favor nonspecific recruitment of T cells as the explanation for the restimulation of Mls-locus blasts by all stimulator cells tested. However, the results using T-cell lines suggest heterogeneity of stimulating antigens in this system, since anti-I-A antibody blocking shows almost complete MHC restriction of this response, while T-cell lines show only partial preference for self-I-A stimulators (Table 10).

The lack of MHC restriction in responses of T blasts primed to Mls-locus antigens, even across full MHC differences, could have an alternative, if less conventional explanation. This would be that T cells differentiate from an MHC-restricted to an MHC-unrestricted state during their activation by Mls-locus antigens. If this were shown to be true it would be strong support for molecular complexity in T-cell receptors, since it would suggest partial loss of a T cell's specificity. Another possibility is that T cells primed to Mls-locus antigens become highly sensitive to TCGF, which is produced by a small number of unrestricted T cells in the secondary response. Finally, the different results of Molnar-Kimber and Sprent (1980) could be explained by their use of T cells from mesenteric lymph node, where a steady influx of antigen from the gut may keep the T cells activated, and hence less restricted. It should be noted that these authors find relatively small responses using mesenteric node cells. Also, there may be a good deal of undetected MHC restriction in their responses to Mls-locus antigens, since in many cases only poor responses to *Mls* presented on allogeneic cells was found. Another possibility

is that *Mls*-locus responses involve synergistic interactions in the primary response between a restricted and a nonrestricted set of cells. However, once primed, the nonrestricted set may be able to respond on their own, rather like memory cells for cytotoxic killer T cells.

Given that the primary response to Mls^a and Mls^d appears to involve recognition of both Mls and I-A antigens, this system may serve as a useful paradigm for the primary in vitro response of Ly1 T cells to antigens associated with I-A-encoded structures. In this regard, it is interesting to note that the data are consistent with the now generally accepted notion that MHC restriction predates the encounter with antigen, and that MHC restriction is learned during the ontogeny of the T cell. Thus, the response to the *Mls* locus may serve as a useful tool in dissecting the significance of MHC restriction, since the response is powerful and readily detected in primary culture.

The most puzzling aspect of the current experiments is the very high frequency of precursor T cells reactive to Mls-locus antigens, and the potency of the response detected. Even without the use of enhancing supernatants (Ryser and MacDonald 1979), it was possible to detect responses with as few as 100 responder cells per well (Figs. 1 and 2) and the frequency of precursors was as great as or greater than that found with MHC differences in these studies. Perhaps the enhancing supernatants used by Ryser and MacDonald (1979) would increase the responses to foreign MHC antigens selectively, leading to greater precursor frequencies for MHC-reactive T cells. However, since our frequencies are not very different from those detected by Ryser and MacDonald (1979), it seems unlikely that our estimate of precursors reactive to Mls^a or Mls^d are very far wrong. While it is possible to explain a high frequency of MHC reactive T cells on the basis of several different hypotheses which attempt to explain MHC restriction (Janeway et al. 1976, Doherty et al. 1977, von Boehmer et al. 1978, Langman 1978), the finding of a high frequency of T cells responding to *Mls*-locus differences would not be predicted. Furthermore, we will probably not understand it until a physiological role for the *Mls* locus has been found. However, it is hard to believe that an antigen stimulating so strong a response by T cells could be present for other than biologically important reasons. The most likely reason would be to mediate T-B or T-macrophage interactions; however, no restrictions mediated by Mls-locus antigens have been observed (Janeway 1976, Katz et al. 1973), although these antigens can provoke allogeneic effects.

In conclusion, the role of the *Mls* locus remains a mystery and an interesting and most certainly significant one at that. The present experiments would seem to clarify two points. One is that naive T cells respond to Mls-locus antigens in association with I-A-encoded structures, and the T cells that do so are Ly1 cells present at a high frequency in normal spleen-cell populations. The further study of this system should reveal interesting findings about the nature of MHC-restriction in Ly1 T cells and the role of Mls-locus antigens on B-cell surfaces.

Acknowledgements. The authors would like to thank Drs. R. Gershon, D. Murphy, L. A. Herzenberg, A. B. Peck, and H. Wigzell for their interest and help with these experiments. The technical assistance of Deborah Bert, Ann Danzey Burnham and Elaine Dzierzak, and the typing of Carol Sanford are gratefully acknowledged. This work was supported by grant AI-15479, and EAL by NIH-MSTP GM-07205. Anti-Ia sera were produced under grant CA 16359.

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Received January 15, 1980; revised version received February 12, 1980