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# T CELL LINES WITH DUAL SPECIFICITY FOR STRONG Mls AND H-2 DETERMINANTS[1]

KATHERINE L. MOLNAR-KIMBER, SUSAN R. WEBB, JONATHAN SPRENT, AND DARCY B. WILSON

*From the Department of Pathology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104*

**To examine the relationship of T cell specificity for Mls *vs* H-2 determinants, BALB/c (*H-2$^d$,Mls$^b$*)(*d,b*) T cells were stimulated repeatedly *in vitro* with H-2-compatible, Mls-incompatible DBA/2 (*d,a*) stimulators. This line of T cells gave strong mixed-lymphocyte reactions to the priming Mls$^a$ determinants but, in addition, gave appreciable responses to various foreign H-2 determinants. When this T cell line was subsequently stimulated over a period of 2 mo with Mls$^a$-negative cells of a particular foreign *H-2* haplotype, e.g., H-2$^k$, the cells gave high responses to H-2$^k$ determinants but only very low responses to third-party H-2 determinants. Significantly, the cells retained high reactivity for Mls$^a$ determinants. In other experiments, BALB/c T cells positively selected to Mls$^{a,d}$-negative H-2-incompatible stimulator cells retained high reactivity for Mls$^a$ determinants. The implications of these findings are discussed.**

It is generally accepted that primary mixed lymphocyte reactions (MLR) of T lymphocytes are directed predominantly to allogeneic gene products of the major histocompatibility complex (MHC)[2] (1). In the mouse, strong MLR also result from stimulation of unprimed T cells by determinants encoded by a different locus, the *M* or *Mls* locus, which is not linked to the *H-2* complex. Four alleles have been described for this locus, i.e., *Mls$^{a,b,c,d}$* (2). Two of these, *b* and *c*, are only weakly stimulatory in primary MLR; the other 2, *a* and *d*, are strongly stimulatory and cause proliferative responses that are as intense as those against MHC determinants. Although *Mls$^{a,d}$* are described as distinct alleles, we (3) and others (4) have found that the Mls$^a$ and Mls$^d$ determinants show cross-reactivity that is so extensive that these 2 gene products may in fact be identical. Recent work from this laboratory has shown that primary MLR to Mls$^{a,d}$ determinants do not exhibit H-2 restriction (3); thus, unlike other non-MHC antigens, these determinants do not seem to be recognized in association with H-2 gene products.

For responses to MHC determinants, the proportion of reactive T cells has been shown to be unusually high ($\approx$5%), a finding that raises the important question as to whether T cells reactive to MHC alloantigens and those reactive to conventional antigens exist as distinct or as overlapping subpopulations (5). In this context, it should be noted that the precursor frequency of T cells reactive to Mls determinants is probably also very high, since primary MLR responses to gene products of the Mls locus are of a magnitude comparable to those against MHC alloantigens (3, and C. A. Janeway, Jr., personal communication). In other respects, however, anti-Mls and anti-MHC responses are quite dissimilar. Thus, unlike MHC determinants, Mls determinants do not evoke cell-mediated lympholysis (6) or lethal graft-*vs*-host disease (7, 8), do not act as transplantation antigens (9, 10), and are not serologically detectable (11, 12). These unusual properties imply that there might be an essential difference in T cell recognition of Mls *vs* MHC determinants. To investigate this question, the present study examines the specificity of T cells stimulated repeatedly *in vitro* with either Mls- or MHC-disparate stimulator cells. The results favor the view that certain T cells have dual specificity both for Mls$^{a,d}$ determinants and for particular MHC determinants.

## MATERIALS AND METHODS

*Mice.* C3H/HeJ, AKR/J, CBA/J, B10.BR, BALB/c, DBA/2, C57BL/6, and SJL mice were purchased from The Jackson Laboratory, Bar Harbor, ME. AKR/Cum mice were obtained from Cumberland View Farms, TN. BALB.B and BALB.K mice were generously provided by Dr. K. Blank, A.BY mice by Dr. W. Silvers, and B10.Q mice by Dr. D. Gasser, all from the University of Pennsylvania School of Medicine. D1.C, D1.LP, B10.RIII, B10.M, and CBA/CaHn mice were bred in our own colony.

*Continuous culture of T cell lines.* Primary mixed lymphocyte bulk cultures were performed as described previously (3). $2 \times 10^7$ lymph node (LN) cells were stimulated with $2 \times 10^7$ allogeneic irradiated (3000 rads) spleen cells in 20 ml EHAA medium supplemented with 0.5% fresh mouse serum. For subsequent restimulations, 5–10 $\times 10^5$ responder cells were cultured with $2 \times 10^7$ irradiated (3000 rads) allogeneic stimulator cells in upright flasks (No. 25110, Corning, N.Y.) in 10 ml supplemented EHAA media; ten percent fetal calf serum was substituted for the 0.5% mouse serum. The cultures were incubated at 37°C in a humidified atmosphere of 7.5% $CO_2$ in air, and were restimulated every 5–14 days. After each restimulation, the proliferative specificity of the T-cell line was analyzed in microtiter plates by culturing various numbers of responder cells with $2.5 \times 10^5$ irradiated (1300 rads) stimulator cells from various donor strains. For these cultures, EHAA medium was supplemented with 2.5% normal rat serum and the proliferative response was assessed by incorporation of tritiated thymidine ($^3$H-TdR).

## RESULTS

*Anti-H-2 reactivity of T cells repeatedly stimulated with Mls$^a$ determinants.* BALB/c (*H-2$^d$,Mls$^b$*) (*d,b*) LN cells were positively selected to Mls$^a$ determinants by repeated stimula-

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[2] Abbreviations used in this paper: MHC, major histocompatibility complex; LN, lymph node; EHAA medium, enriched high amino acid medium; $^3$H-TdR, tritiated thymidine.



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tion of the cells with H-2-compatible, Mls-different DBA/2 (*d,a*) spleen cells in bulk culture. Table I shows the specificity of these BALB/$c_{+DBA/2}$ T (>98% Thy 1.2 positive) cells in MLR determined after the first (day 14), third (day 41), and fifth (day 60) stimulation. As expected, strong responses were observed against stimulators expressing the priming $Mls^a$ (or cross-reactive $Mls^d$) determinants (group B); in accordance with our previous findings (3), these responses were not H-2 restricted. The surprising finding, however, was that appreciable responses were also observed against various $Mls^{a,d}$-negative cells expressing foreign H-2 determinants, e.g., $H\text{-}2^b$, $H\text{-}2^k$, $H\text{-}2^s$, $H\text{-}2^r$, and $H\text{-}2^f$ (group C, Table I, and see Table I footnote). With regard to the possibility that these anti-H-2 responses reflected a significant degree of contamination with bystander cells, the following points should be made: 1) After the first bulk culture, each restimulation resulted in a 5- to 10-fold increase in cell numbers, and any bystanders should have been diluted by a factor of >2500 by day 60 (assuming that cells were not recruited nonspecifically). 2) The possibility that bystander cells were "back-stimulated" by T cells in the stimulator population is unlikely, since equivalent cell yields and MLR occurred when anti-Thy 1.2 serum plus complement-treated stimulator cells were used (data not shown). 3) Syngeneic BALB/c stimulators did not yield significant cell growth in bulk cultures. 4) The magnitude of the anti-H-2 responses showed no sign of decreasing with each restimulation against $Mls^a$-bearing cells.

None of the above points constitute *prima facie* evidence against the possible involvement of bystander cells. For this reason, it was decided to divert the T cell line by stimulating the bulk cultures, not with $Mls^a$-bearing stimulators, but with Mls-compatible, H-2-different CBA/CaHn (*k,b*) or C57BL/6 (*b,b*) cells. Each restimulation with H-2-different cells resulted in a decreased proliferative response to third-party H-2 determinants but, significantly, did not reduce responses to $Mls^a$ determinants. Table II shows the specificity of BALB/c LN cells stimulated twice with DBA/2 cells (days 0 to 27), then 7 times with C57BL/6 (*b,b*) cells (days 27 to 892), and finally once (Expt. 1) or 3 times (Expt. 2) with H-2 congenic BALB.B(*b,b*) cells (days 82 to 99); the latter cells were used to avoid responses to minor H antigens. Despite the fact that the T cells had not been exposed to $Mls^a$ determinants for a period of 60 days (72 days for Expt. 2), it is apparent that they nevertheless retained the capacity to give strong proliferative responses to a panel of stimulator cells expressing $Mls^{a,d}$ determinants. With $Mls^{a,d}$-negative stimulators, the cells gave good responses against $H\text{-}2^b$ determinants, intermediate responses to $H\text{-}2^k$, and very low or undetectable responses to $H\text{-}2^{r,f,s,q}$. Likewise, BALB/c LN cells stimulated twice with DBA/2 and then 8 to 10 times with $H\text{-}2^k$ [CBA/CaHn 5 times and BALB.K 3 times (Expt. 1) or 5 times (Expt. 2)] retained high reactivity to $Mls^{a,d}$ and to $H\text{-}2^k$ but gave low or insignificant responses to $H\text{-}2^{b,r,f,s,q}$.

TABLE I

*Specificity of BALB/c LN cells in MLR after repeated stimulation with DBA/2 stimulator cells in bulk culture*

| Group | Stimulators | (*H-2,Mls*) | $^3$H-TdR Uptake (cpm × $10^{-2}$) at:[a] | | |
|---|---|---|---|---|---|
| | | | 14 days | 41 days | 60 days |
| A | BALB/c | (*d,b*) | 1.2 ± 0.1[b] | 3.6 ± 2.0 | 5.8 ± 0.7 |
| B | DBA/2 | (*d,a*) | 158.1 ± 38.9 | 242.5 ± 12.8 | 76.6 ± 7.9 |
| | D1.C | (*d,a*) | 75.9 ± 10.5 | 290.8 ± 12.0 | 72.0 ± 5.2 |
| | D1.LP | (*b,a*) | 51.1 ± 3.6 | 243.4 ± 11.4 | 65.4 ± 2.2 |
| | AKR/Cum | (*k,a*) | 73.5 ± 7.2 | ND | ND |
| | AKR/J | (*k,a*) | 99.4 ± 25.0 | 209.2 ± 8.6 | 57.7 ± 10.6 |
| | CBA/J | (*k,d*) | 132.4 ± 37.4 | ND | ND |
| C | C57BL/6 | (*b,b*) | 9.4 ± 1.2 | 104.3 ± 20.2 | 59.9 ± 14.9 |
| | CBA/CaHn | (*k,b*) | ND[c] | 40.7 ± 17.8 | 54.2 ± 12.1 |
| | C3H/HeJ | (*k,c*) | 26.0 ± 0.7 | ND | ND |
| | SJL | (*s,c*) | 7.7 ± 3.2 | 27.3 ± 4.0 | 27.4 ± 12.2 |

[a] At time of harvesting BALB/c cells from bulk cultures. Proliferative response of 25 × $10^3$ (14 days), 6 × $10^3$ (41 days), and 6 × $10^3$ (60 days) BALB/$c_{+DBA/2}$ T cells, respectively, measured on day 2. In other experiments, high proliferative responses were also observed to B10.RIII (*H-2$^r$,Mls$^b$*), and B10.M (*H-2$^f$,Mls$^b$*).

[b] Mean cpm ± SD of 3 replicate cultures.

[c] Not determined.

TABLE II

*Specificity of BALB/c LN cells in MLR after repeated stimulation in bulk cultures with a) an Mls difference (Mls$^a$) followed by b) an H-2 difference (H-2$^b$ or H-2$^k$)*

| Group | Stimulators | (*H-2, Mls*) | $^3$H-TdR Uptake (cpm × $10^{-2}$): Secondary stimulations with BALB.B (*b,b*)[a] | | Secondary stimulations with BALB.K (*k,b*)[a] | |
|---|---|---|---|---|---|---|
| | | | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 |
| A | BALB/c | (*d,b*) | 0.7 ± 1[b] | 2.2 ± 0.1 | 0.7 ± 0.1 | 2.7 ± 0.9 |
| B | DBA/2 | (*d,a*) | 23.2 ± 2.2 | 30.8 ± 1.4 | 36.6 ± 4.9 | 70.6 ± 13.3 |
| | D1.C | (*d,a*) | 27.6 ± 2.4 | 13.8 ± 1.4 | 46.7 ± 4.8 | 39.6 ± 5.2 |
| | D1.LP | (*b,a*) | 23.5 ± 1.2 | 21.4 ± 4.0 | 23.9 ± 2.2 | 35.7 ± 4.7 |
| | AKR/Cum | (*k,a*) | ND | 13.5 ± 0.1 | ND | 38.5 ± 5.2 |
| | CBA/J | (*k,d*) | 17.7 ± 0.4 | 19.8 ± 0.4 | 34.2 ± 2.2 | 52.7 ± 4.1 |
| C | C57BL/10 | (*b,b*) | 13.2 ± 4.3 | 11.4 ± 0.3 | 3.6 ± 0.8 | 3.4 ± 0.8 |
| | BALB.B | (*b,b*) | 12.0 ± 0.7 | 65.5 ± 7.0 | 1.6 ± 0.2 | 9.6 ± 0.5 |
| | A.BY | (*b,c*) | 25.4 ± 2.6 | 15.2 ± 1.7 | 4.1 ± 0.7 | 3.7 ± 1.0 |
| | CBA/CaHn | (*k,b*) | 6.9 ± 0.6 | 8.2 ± 0.4 | 27.4 ± 1.5 | 24.3 ± 2.3 |
| | B10.BR | (*k,b*) | 6.5 ± 0.5 | 2.8 ± 0.6 | 24.1 ± 5.0 | 12.1 ± 0.9 |
| | BALB.K | (*k,b*) | 8.0 ± 0.6 | 5.0 ± 0.9 | 21.4 ± 3.0 | 25.1 ± 3.6 |
| | C3H/HeJ | (*k,c*) | ND | 6.3 ± 0.1 | ND | 12.7 ± 0.4 |
| | B10.RIII[c] | (*r,b*) | 2.4 ± 0.7 | 1.9 ± 0.2 | 0.9 ± 0.1 | 2.3 ± 0.9 |
| | B10.M[c] | (*f,b*) | 0.4 ± 0.1 | 2.2 ± 0.7 | 0.8 ± 0.1 | 2.9 ± 1.3 |
| | B10.S[c] | (*s,b*) | 0.5 ± 0.1 | 2.5 ± 0.5 | 0.6 ± 0.1 | 3.7 ± 1.1 |
| | B10.Q[c] | (*q,b*) | 1.3 ± 0.3 | 2.4 ± 0.1 | 1.9 ± 0.4 | 2.0 ± 0.4 |

[a] Proliferative response of 2.5 × $10^4$ BALB/$c_{+DBA/2}$ T cells restimulated repeatedly with BALB.B or BALB.K cells, respectively, as measured on day 2; see text for details.

[b] Mean cpm ± SD of 3 replicate cultures.

[c] In the same experiment, cells from these strains effectively stimulated normal BALB/c and CBA/CaHn LN cells.

***Anti-Mls $^a$ reactivity of T cells selected to H-2 determinants.*** In reciprocal experiments, BALB/c LN cells were stimulated 4 times with H-2-different, Mls-compatible C57BL/6 (*b,b*) cells over a period of 51 days and then tested in MLR. As shown in Table III, the cells responded well against the priming $H\text{-}2^b$ determinants but poorly to third-party $H\text{-}2^k$ and $H\text{-}2^a$ determinants. Significantly, the cells retained the capacity to give high responses against 4 stimulator populations expressing $Mls^a$ determinants (group B).

## DISCUSSION

At face value, the finding that T cells stimulated repeatedly with $Mls^a$ determinants retain reactivity to particular H-2 determinants, and *vice versa*, would seem to argue strongly that

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TABLE III

*Specificity of BALB/c LN cells in MLR after stimulation with C57BL/6 cells 4 times over a period of 51 days*

| Group | Stimulators | (*H-2,Mls*) | $^3$H-TdR Uptake (cpm × $10^{-2}$)[a] |
|---|---|---|---|
| A | BALB/c | (*d,b*) | 0.7 ± 0.3[b] |
| B | D1.C | (*d,a*) | 19.9 ± 2.0 |
| | DBA/2 | (*d,a*) | 58.0 ± 3.2 |
| | D1.LP | (*b,a*) | 58.9 ± 2.2 |
| | AKR/J | (*k,a*) | 14.7 ± 0.8 |
| C | C57BL/6 | (*b,b*) | 36.9 ± 6.2 |
| | CBA/CaHn | (*k,b*) | 2.7 ± 0.9 |
| | SJL | (*s,c*) | 5.0 ± 1.3 |

[a] Proliferative response of 3 × $10^3$ BALB/$c_{+C57BL/6}$ T cells measured on day 2.

[b] Mean cpm ± SD of replicate cultures.

certain T cells have joint specificity for these 2 sets of determinants. Participation of bystander cells seems unlikely because repeated stimulation with particular H-2 determinants led to a marked loss of reactivity to third-party H-2 determinants (Tables II, III); one would have to postulate that nonspecific recruitment of bystanders applies only to Mls-reactive cells and not to H-2-reactive cells. In addition, the proliferation of putative bystander Mls-reactive cells would need to have been as intense as that of the H-2-specific cells because reactivity to Mls was not diminished in the allostimulated lines. Formal evidence against the participation of bystanders, however, will require studies on cloned lines of cells. In this respect, recent preliminary experiments with certain cloned lines of T cells indicate that they do appear to have joint specificity in MLR for $Mls^a$ and particular H-2 determinants.

In interpreting the data, it should be emphasized that the cell lines in Table III could be triggered *either* by Mls determinants *or* by H-2 determinants alone. Such a finding would seem to exclude the simple possibility that the T cell lines recognized as association of Mls plus H-2 determinants, either via 1 receptor (for neoantigenic determinants) or via 2 receptors. With this constraint in mind, we can offer 2 possible explanations for the apparent dual specificity of T cells for Mls and H-2 determinants. First, Mls determinants may be recognized by a unique set of receptors distinct from those that recognize either MHC determinants or conventional antigens. Such receptors might be analogous to surface structures that recognize T cell mitogens. Alternatively, one might argue that Mls determinants comprise a variety of epitopes, each of which cross-reacts with a particular MHC determinant. In this case, both sets of determinants would be recognized by the same repertoire of receptors. It should be added that since T cells from $Mls^a$ bearing strains do not show a perceptible reduction in overall anti-H-2 reactivity, one must postulate that only a minor proportion of H-2 determinants of each haplotype could show cross-reactivity with Mls epitopes.

It should be pointed out that the existence of T cells specific for both H-2 and Mls determinants does not preclude the existence of cells specific for only 1 of these 2 sets of determinants. Indeed, it is quite clear that many H-2-specific clones do not have detectable Mls reactivity (13). Likewise, only a proportion of H-2-reactive T cells possess Mls reactivity since T cells depleted of reactivity to $Mls^{a,d}$ determinants give high anti-H-2 responses (3).

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