Brief Definitive Report

T CELL RECEPTORS FOR RESPONSES TO Mls DETERMINANTS AND ALLO-H-2 DETERMINANTS APPEAR TO BE ENCODED ON DIFFERENT CHROMOSOMES

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In the mouse, two sets of determinants induce strong T cell proliferative responses in primary mixed lymphocyte culture: allogeneic H-2 determinants encoded by genes of the major histocompatibility complex (MHC) located on chromosome 17, and determinants encoded by the minor lymphocyte stimulating (*Mls*) locus on chromosome 1 (1). Unlike MHC molecules, Mls determinants are undetectable serologically, do not evoke typical transplantation reactions, do not appear to act as targets for cytotoxic T cells, and show only limited polymorphism (reviewed in 2). Of the determinants of the four described *Mls* alleles, *a*, *b*, *c*, and *d*, only Mls^a and Mls^d determinants elicit strong responses; these two sets of determinants cross-react extensively, and, indeed, are indistinguishable in our hands. In contrast to conventional antigen, T cell responses to Mls determinants do not exhibit classic H-2 restriction (2).

Recently (2), we reported that $Mls^{a,d}$-specific T cell clones concomitantly display apparently random specificity for allo-H-2 determinants. Given this, and the odd properties of Mls determinants (see above), we suggested that T cell recognition of these determinants might be controlled by a separate set of receptors, unrelated to the receptors that recognize conventional antigens (self plus X) or allo-H-2 antigens. In support of this, we show that T cell hybridomas with dual reactivity for $Mls^{a,d}$ and allo-H-2 can lose reactivity to allo-H-2 while retaining reactivity to $Mls^{a,d}$, and vice versa. Thus, receptors for $Mls^{a,d}$ and allo-H-2 determinants are probably encoded on different chromosomes.

Materials and Methods

Mice.   BALB/c, B10.D2, DBA/2, CBA/J, and B10.BR mice were all obtained from The Jackson Laboratory, Bar Harbor, ME. B10.RPD2 mice were purchased through Dr. Chella David, Mayo Clinic. B10.P mice were provided by Dr. J. Stimpfling, McLaughlan Research Institute and Dr. D. Sachs, National Institutes of Health.

T Cell Clones.   T cell clones displaying dual reactivity for Mls and H-2 were derived and characterized as previously described (2). Briefly, B10.D2 ($H-2^d$, Mls^b) lymph node (LN) cells were stimulated in vitro with irradiated (3,000 rad) DBA/2 ($H-2^d$, Mls^a) spleen

cells. 14 d later the viable cells were restimulated with irradiated DBA/2 spleen cells; 24 h later they were cloned at limiting dilution in flat-bottom microtiter plates containing 5 × 10⁵ irradiated B10.RPD2 (*I-A^p*, *Mls^b*) spleen cells. The clones were expanded at 2-wk intervals in culture medium (RPMI 1640 plus 10% fetal calf serum) containing supernatant (SN) from 36-h concanavalin A (Con A)–stimulated rat spleen cell cultures. D10.2, the T cell clone used to generate one set of hybridomas (see below), proliferates in response to all Mls^{a,d}-positive strains tested, including DBA/2, CBA/J, NZB, AKR/J, D1.LP, and SM/J. The clone has alloreactivity to H-2^P but does not respond to cells from any of nine Mls^{a,d}-negative strains tested of various *H-2* haplotypes and backgrounds.

Fluorescence-activated Cell Sorter (FACS) Analysis.   Viable hybridoma cells were incubated with culture supernatant of the GK1.5 B cell hybridoma (3) (provided by F. Fitch, University of Chicago) and then with fluorescein isothiocyanate (FITC)-F(ab')₂ sheep anti–rat Ig (Cappel Laboratories, Cochranville, PA) reagent. The cells were analyzed on a FACS IV (B-D FACS Systems, Sunnyvale, CA) by Dr. Alan Pickard, University of Pennsylvania.

T Cell Hybridomas.   The T cell hybridoma FS11.155 was prepared by fusion of Con A–stimulated T cell blasts from a B10.Q (*H-2^q*, *Mls^b*) mouse spleen to the T cell tumor, BW5147, by previously described methods (4). This hybridoma responded to spleen cells from all Mls^{a,d}-positive strains tested (DBA/2, CBA/J, NZB, AKR/J, D1.LP, and SM/J). The second hybridoma described, BD10.42, was the product of a fusion of the T cell clone, D10.2 (described above), to BW5147 using the same procedures. Subclones of hybridomas were prepared by recloning at limiting dilution.

Interleukin 2 (IL-2) Assay.   Using standard procedures (4), hybridomas (1–2 × 10⁵ cells) were assayed for their ability to produce IL-2 when stimulated with 5 × 10⁵ irradiated (1,500 rad) spleen cells bearing the appropriate MHC or *Mls* haplotype. 24–48 h later, 100 μl of medium was removed and added to 100 μl medium containing 4,000 HT.2 cells (5) or CTLL-2-15H cells (derived originally by Drs. P. Baker, S. Gillis, and K. Smith, Dartmouth Medical School); we used these only once (line 1 of Table I). The cultures were pulsed 24 h later with [³H]thymidine, harvested, and counted 18 h afterwards. With a 1:4 dilution of rat Con A SN, HT-2 or CTLL cells incorporated 60,000–100,000 cpm. Cultured alone, these indicator cells took up <500 cpm.

Results

Experimental Approach.   T cell hybridomas are karyotypically unstable and lose chromosomes for some period of the time after fusion (6). Hence, if the recognition structures for T cell responses to Mls and H-2 determinants were encoded on the same chromosome, then loss of reactivity to Mls determinants by chromosome loss should also be accompanied by loss of H-2 responsiveness, and vice versa. Conversely, if these receptor structures were encoded on different chromosomes, then some hybridoma clones should show Mls reactivity but lack H-2 reactivity, and vice versa.

To test these predictions we prepared two different T cell hybridomas with dual specificity for Mls^{a,d} and allo-H-2 determinants. The hybridomas were repeatedly subcloned by limiting dilution. After each round of cloning 30–100 hybrids were tested for their ability to secrete IL-2 when triggered with Mls- or with H-2-disparate stimulator cells.

Loss of Mls Reactivity.   The first hybridoma, FS11.155, showed strong reactivity in IL-2 assays to H-2^d stimulators and weaker, but significant responses to Mls^{a,d} stimulators (Table I). Subclones of this hybridoma showed three different reactivity patterns, represented in Table I. Some (e.g., FS11.155.14) responded well to both Mls^{a,d} and H-2^d, some (e.g., FS11.155.16) responded only to H-2^d but lacked reactivity to Mls^{a,d} (Table I), and others (e.g., FS11.155.12) reacted with neither H-2 nor Mls determinants.

TABLE I

Specificity of Subclones of Dual-reactive Hybridoma FS11.155 in IL-2 Assay

| Hybridoma | Stimulators | | |
	B10.BR $(\text{H-2}^k, \text{Mls}^b)$	CBA/J $(\text{H-2}^k, \text{Mls}^d)$	B10.D2 $(\text{H-2}^d, \text{Mls}^b)$
	cpm		
FS11.155	496	1,149	14,025
FS11.155.14*	261	29,232	28,825
FS11.155.12*	198	111	308
FS11.155.16*	226	229	16,438

For details of IL-2 assay, see Materials and Methods.
* Subclones were picked from limiting dilution plates that had 36 out of 96 wells positive for growth.

TABLE II

Specificity of Subclones of FS11.155.14 in IL-2 Assay

| Subclone* | Stimulators | | |
	B10.BR $(\text{H-2}^k, \text{Mls}^b)$	CBA/J $(\text{H-2}^k, \text{Mls}^d)$	BALB/c $(\text{H-2}^d, \text{Mls}^b)$
	cpm		
FS11.155.14.7	98	33,364	24,194
FS11.155.14.23	667	237	277
FS11.155.14.37	240	155	20,369

See Table I.
* Subclones shown were picked from plates that had 24 out of 96 wells positive for growth.

The dual-reactive hybridoma clone FS11.155.*14* was further subcloned; 23 of 39 subclones maintained dual reactivity for Mls and H-2, four of 39 lacked both reactivities, and 10 of 39 could be stimulated only with H-2^d stimulators.[1] Representative subclones of these three groups are described in Table II. Fig. 1 summarizes the results obtained with cloning of the FS11.155 hybridoma. Two points seem evident: (a) the proportion of subclones showing only H-2 responses dropped from ~50% (15/33) in the first round of cloning to 5% (3/65) in the third round; while (b) the proportion of dual-reactive clones increased from 50% (14/33) to ~85% (54/65). Evidently, the FS11.155 hybridoma became more stable karyotypically during the process of sequential subcloning.

Loss of H-2 Reactivity.   The second hybridoma, BD10.42, was obtained from a fusion of BW5147 with a T cell clone, D10.2, expressing dual specificity for $\text{Mls}^{a,d}$ and H-2^p (Materials and Methods). At the time of fusion, D10.2 (a subclone of parental clone D10) was further subcloned; all 20 subclones showed dual reactivity for $\text{Mls}^{a,d}$ and H-2^p. After fusion, the hybridoma BD10 proved to be so unstable that three rounds of sequential subcloning were required to derive a definitive assessment of specificity in IL-2 assays. Typical specificity patterns that emerged after these repeated subclonings are shown in Table III. Many of the

[1] Two further subclones gave low responses to H-2^d and no response to $\text{Mls}^{a,d}$. Little emphasis can be placed on these findings, however, because further subcloning yielded hybridomas that were all negative on both $\text{Mls}^{a,d}$ and H-2^d.

FS11.155

| H-2 +, Mls − | H-2 +, Mls + | H-2 −, Mls − | 1st Subcloning |
| (15/33) | (14/33) | (4/33) | |

| H-2 +, Mls − | H-2 +, Mls + | H-2 −, Mls − | 2nd Subcloning |
| (10/39) | (23/39) | (4/39) | |

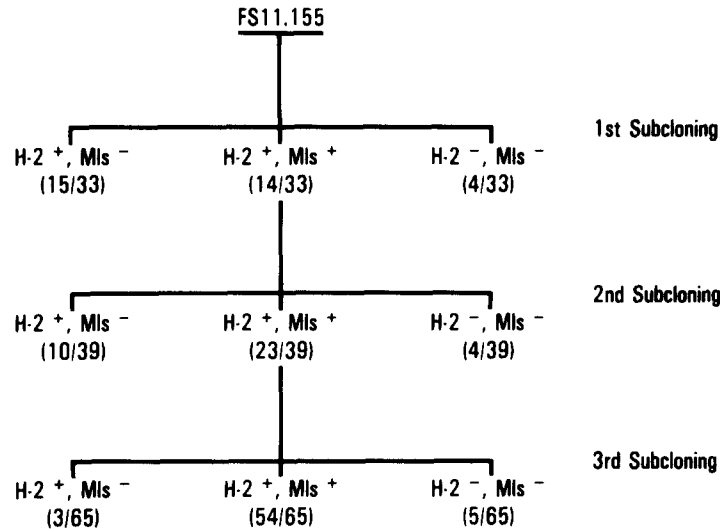| H-2 +, Mls − | H-2 +, Mls + | H-2 −, Mls − | 3rd Subcloning |
| (3/65) | (54/65) | (5/65) | |

FIGURE 1. The data summarizes the specificity patterns observed after repeated subcloning of the H-2^d, Mls^{a,d}–reactive hybridoma FS11.155. After each round of subcloning, hybridomas retaining dual specificity for H-2^d and Mls^{a,d} were further subcloned. Two subclones in the second round of subcloning and three hybridomas in the third round were not easy to categorize, and have been omitted from the data presented.

TABLE III

Reactivity Patterns of BD10.42 Subclones in IL-2 Assay

| | | Stimulators | | |
Exp.	Hybridomas	B10.BR (H-2^k, Mls^b)	CBA/J (H-2^k, Mls^d)	B10.P (H-2^P, Mls^b)
			cpm	
1	BD10.42.20.65	386	26,762	4,285
	BD10.42.9.5.7	76	47,500	45
2	BD10.42.9.5.7*	40	28,472	81
	.8	117	7,447	44
	.9	43	13,355	96
	.10	49	22,336	55
	.11	37	18,681	56
	FD13.4.3.11a3‡	116	29,646	16,600

See Table I.

* Subclones were picked from plates that had 18 out of 96 wells positive for growth. The other subclones tested had a similar reactivity pattern.

‡ FD13.4.3.11a3 is an independently derived T cell hybridoma used in this experiment as a positive control for the B10.P stimulator cells.

subclones (e.g., BD10.42.20.65) reacted with both Mls^{a,d} and H-2^P. Others responded to neither stimulator (not shown). Some, like BD10.42.9.5, showed no reactivity to H-2^P and strong reactivity to Mls^{a,d}; the specificity patterns of five representative subclones derived from BD10.42.9.5 are shown in Table III. The clones exemplified in Table III showed the same specificity pattern in four separate experiments performed over a 6-mo period.

Expression of the L3T4 Marker. The mouse T cell surface molecule L3T4, defined by monoclonal antibody GK1.5 (3), is thought to be involved in raising

the avidity with which T cells react with antigen (7). It was therefore of interest to know whether the loss of only one of the two antigen specificities by the T hybridomas would correlate with the loss of the L3T4 molecule. No evidence could be found for such an association. Both sets of hybridomas (FS11.155 and BD10.42) were initially L3T4$^+$, as assessed by FACS analysis with the antibody GK1.5 (data not shown). All of the BD10.42-derived hybridomas retained the expression of L3T4, despite repeated subcloning. In marked contrast, all of the FS11.155-derived hybridomas were negative for L3T4 after the third subcloning, irrespective of their specificity. Loss of the L3T4 marker is thus an unlikely explanation for the above results.

Discussion

Using karyotypically unstable T cell hybridomas, we demonstrate here that dual-reactive hybridomas specific for Mls^{a,d} and allo-H-2 determinants generate variants which no longer respond to one of these sets of stimulating determinants, but continue to respond to the other set. The simplest explanation for this observation is that Mls and H-2 determinants are recognized by different sets of receptor structures, encoded on different chromosomes. In this case, loss of the chromosome that encodes receptors with anti-Mls reactivity would not affect anti-H-2 reactivity, and vice versa.

Alternatively, one could postulate some mutational event that modifies the receptor only slightly, such that it will still react with one ligand but not the other. This mutation would have to have several unusual characteristics. For example, it would have to occur with an unusually high frequency during the first 1–2 mo after fusion, since each round of subcloning during this time generated a significant proportion of new clones lacking one reactivity. The mechanism operative in generating such mutations would then cease within a few months after fusion, since after this time we were rarely able to find new clones lacking one reactivity. The majority of the subclones obtained from late cloning rounds either maintained both reactivities or lost both. Those which lost both reactivities were rather frequent, perhaps reflecting defective synthesis or secretion of IL-2. Dual-reactive hybridomas derived soon after fusion often show reactivity patterns in which there is high reactivity to one set of determinants, and intermediate or low reactivity to the other (e.g., FS11.155 in Table I). Although such findings are clearly suggestive of mutation, to date, all attempts to obtain stable hybrids with this specificity have failed. In our hands, further subcloning of the hybridomas invariably generates a mixture of two reactivity patterns (in addition to nonreactive hybridomas): some hybridomas show high reactivity to both sets of determinants, while others react only to one and not the other sets of determinants (see Table I).

For the above reasons, and because of the current lack of evidence for somatic mutation in T cell receptors (8), chromosome loss would seem to be the most likely explanation for the data. Mutations certainly cannot be ruled out, however, and it is quite possible that chromosome loss and/or somatic mutation occur. A clear distinction between the above two explanations must await a more detailed characterization of T cell receptors at the molecular level.

One question raised by these results is why only loss of Mls reactivity occurred with the FS11.155 hybridoma and only H-2 reactivity was lost with the BD10.42 hybridoma. This result may indicate the occurrence of predisposing chromo-

somal events in the immediate postfusion period that dictate which chromosomes can be subsequently lost without compromising the viability of the cell, or its capacity to synthesize and secrete IL-2.

If recognition of Mls determinants is indeed controlled by a separate set of receptors, what is the biological significance of Mls determinants? Although several possibilities have been suggested (9, 10), our current view is that, since Mls molecules are expressed predominantly on B cells, Mls molecules might function as accessory structures on B cells, increasing the overall avidity of T cells reactive with antigen on these cells, or perhaps allowing the production of a B cell lymphokine.

Summary

Previous studies have shown that T cell clones specific for strong Mls[a,d] determinants concomitantly display apparently random reactivity to allo-H-2 determinants. One explanation for this finding is that T cell recognition of Mls[a,d] and allo-H-2 determinants is controlled by separate sets of receptors. If these receptors were chromosomally unlinked, karyotypically unstable T cell hybrids with dual reactivity for Mls[a,d] and particular allo-H-2 determinants would be expected, occasionally, to lose reactivity for one set of determinants, but not the other. The results presented here provide direct support for this prediction.

References

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