Brief Definitive Report

RESPONSE OF MATURE UNPRIMED CD8$^+$ T CELLS TO Mls^a DETERMINANTS

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Murine T cells give high primary proliferative responses to Mls determinants (1). Polymorphism of Mls determinants is very limited and only Mls^a and Mls^c are known to be strongly immunogenic for T cells; Mls^b is nonstimulatory.

In terms of Vβ TCR expression, Mls^a mice show selective deletion of Vβ6$^+$ and Vβ8.1$^+$ T cells, whereas Mls^c mice delete Vβ3$^+$ T cells (1-4). Since anti-Mls responses are strongly inhibited by anti-H-2 class II (Ia) antibodies (5-7) and are reported to be controlled almost entirely by Ia-restricted CD4$^+$ cells (1, 5, 7), most groups have assumed that the T cell TCR-α/β recognizes Mls determinants complexed with Ia molecules. Mature class I-restricted CD8$^+$ cells, by contrast, are considered to display no demonstrable responsiveness to Mls determinants. In fact, some workers argue that CD8 molecules actively inhibit anti-Mls responses (8).

Since it is generally assumed that CD8$^+$ cells display only limited reactivity to class II molecules (but see Discussion), the unresponsiveness of CD8$^+$ cells to Mls determinants is not unexpected. Nevertheless, it is of interest that Vβ6 and Vβ8.1 expression in Mls^a-negative mice is as high on CD8$^+$ cells as on CD4$^+$ cells (2, and see Results). It is also notable that the deletion of Vβ6$^+$ and Vβ8.1$^+$ T cells in Mls^a-positive mice applies to CD8$^+$ cells, as well as to CD4$^+$ cells (2, and see Results). These two sets of observations, together with the isolated report that a cytotoxic CD8$^+$ clone gave proliferative responses to Mls^a stimulator cells (9), stimulated us to reexamine the issue of whether mature CD8$^+$ cells display Mls^a reactivity. The data in this paper demonstrate that the proliferative response of unprimed T cells to Mls^a determinants does involve CD8$^+$ cells, as well as CD4$^+$ cells. The anti-Mls^a response of CD8$^+$ cells is Vβ specific and appears to depend on help from CD4$^+$ cells. These findings indicate that recognition of Mls^a determinants is not a property unique to CD4$^+$ cells.

Materials and Methods

Mice. BALB/c and DBA/2 mice were purchased from Bantin and Kingman, Inc., Fremont, CA. All other mice were bred at Scripps Clinic.

Purification of Cells. Using established techniques (10), T cells were purified from lymph node (LN) suspensions by passage through nylon wool columns followed by treatment with

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anti-I-A plus J11d mAb and C to remove B cells. Purified CD8$^+$ cells were prepared by treating nylon wool-passed T cells with anti-I-A, anti-CD4, and J11d mAbs, plus C followed by positive panning on anti-CD8 mAb-coated plates (10).

Mixed Lymphocyte Reactions (MLR). Using standard conditions (10), primary MLR were set up in 200-μl volumes with 1-2 $\times$ 10^5 responder cells and 5 $\times$ 10^5 stimulator cells (T cell-depleted spleen cells treated with mitomycin C). Cultures were pulsed with 1 μCi [^{3}H]TdR 18 h before harvest. For phenotypic analysis of blast cells, 5 $\times$ 10^6 purified T cells or CD8$^+$ cells were cultured in 2-ml wells with 5 $\times$ 10^6 spleen stimulators (see above) for 4 d; the CD8$^+$ cells were supplemented with rIL-2 (5 U/ml). Blast cells were purified on Ficoll-Hypaque density gradients before FACS analysis.

FACS Analysis. As described elsewhere (11), aliquots of cells were incubated with rat mAb specific for Thy-1 (T24), CD8 (3.168), Vβ8.1+8.2 (KJ16), Vβ6 (RR47), or Vβ11 (RR315), followed (after washing) by FITC-labeled mouse anti-rat Ig mAb. After incubation with rat serum and further washing, CD4 expression was detected with phycoerythrin-labeled anti-CD4 (GK1.5) mAb. To detect Vβ8.2 expression, cells were incubated with mouse anti-Vβ8.2 (F23.2) mAb followed by FITC-labeled goat anti-mouse IgG antibody. Labeled cells were analyzed on a FACS IV flow cytometer using two-channel immunofluorescence. Vβ8.1$^+$ cells were calculated by subtraction (percent of Vβ8.1 + 8.2$^+$ cells minus percent of Vβ8.2$^+$ cells). Selective gating based on light scatter was used for analysis of blast cells.

Results

In Vitro Experiments. Initial studies indicated that purified CD8$^+$ cells from Mls^b mice gave no demonstrable MLR to Mls^a or Mls^c stimulators in the absence of added lymphokines. A typical experiment is illustrated in Table I. Here, it can be seen that, unlike unseparated T cells, purified B10.D2 (Mls^b) CD8$^+$ cells failed to respond to BALB/c (Mls^c) or DBA/2 (Mls^{a,c}) stimulators in the absence of IL-2, but responded well to H-2-different B10.P stimulators. Supplementing CD8$^+$ cells with IL-2 substantially increased the background response with syngeneic stimulators but led to two- to fourfold higher responses with Mls-different stimulators than with syngeneic stimulators. To examine whether the response of CD8$^+$ cells + IL-2 to Mls-different stimulators is directed to Mls determinants, per se, rather than to other cell surface molecules, MLC were harvested on day 4 and stained for Vβ expression (Table II).

When unseparated Mls^b T cells were cultured with Mls-identical H-2-different (class I + II-different) stimulators, CD8$^+$ blasts outnumbered CD4$^+$ blasts by ~2:1 (Table II, Exp. 1, line *2*). With Mls-different H-2-compatible stimulators, by contrast, CD4$^+$ blasts were considerably more numerous than CD8$^+$ blasts. CD8$^+$ blasts were clearly detectable, however, and accounted for 20-25% of the blasts in the two experiments shown (Table II, Exp. *1*, line *1*; Exp. 2, line *1*). Significantly, a high proportion of the CD8$^+$ blasts generated against Mls^{a,c} differences were Vβ6$^+$, i.e., 35-45%; this compared with 55-65% Vβ6$^+$ cells for CD4$^+$ blasts. For blast cells generated against an H-2 difference (Table II, Exp. *1*, line *2*), a much lower proportion of blast cells were Vβ6$^+$.

When CD8$^+$ blasts were generated from purified CD8$^+$ responders cultured with Mls^{a,c} or Mls^a stimulators supplemented with IL-2, 40-60% of the blasts were Vβ6$^+$ (Table II, Exp. *1*, line *3*; Exp. *2*, line *2*; Exp. *3*, line *1*). A much lower proportion of Vβ6$^+$ blasts, i.e., 5-15%, was seen for CD8$^+$ blasts stimulated against an H-2 difference (Table II, Exp. *1*, line *5*; Exp. *3*, lines *2* and *4*) or an Mls^c difference (Table II, Exp. *1*, line *4*). Intermediate levels of Vβ6 blasts (15-30%) were observed

TABLE I
Primary MLR of Unseparated T Cells vs. Purified CD8⁺ Cells to Mls^a-positive Spleen Stimulators

Responder cells	r-IL-2 added to culture	Day of assay	[^{3}H]TdR incorporation with stimulators: B10.D2 (H-2^d,Mls^b)	DBA/2 (H-2^d,Mls^{a,c})	BALB/c (H-2^d,Mls^c)	B10.P (H-2^p,Mls^b)
			cpm × 10^3			
B10.D2 T	–	3	1.0	28.8	1.6	16.3
	–	4	1.9	64.8	3.2	36.2
	+	3	3.8	38.3	8.1	20.5
	+	4	7.2	114.5	20.1	61.5
B10.D2 CD8$^+$	–	3	0.2	0.3	0.4	7.0
	–	4	0.2	0.6	0.6	33.1
	+	3	3.0	9.3	5.3	12.3
	+	4	8.4	19.7	12.2	62.6

Purified T cells or CD8$^+$ cells were prepared from LN suspensions (Materials and Methods). Doses of 10^5 responder cells and 5×10^5 stimulator cells (T cell-depleted mitomycin C-treated spleen) were cultured with or without rIL-2 (5 U/ml) for 3 or 4 d and pulsed with [^{3}H]TdR 18 h before harvest. The data show mean responses of triplicate cultures.

for CD8$^+$ cells cultured with a combined Mls^a + H-2 difference (Table II, Exp. *2*, line *3*; Exp. *3*, line *3*). In contrast to Vβ6, the expression of Vβ8.1 + 8.2 detected by KJ16 mAb showed little variation on the various blasts tested.

In Vivo Experiments. To examine the response of CD8$^+$ cells to Mls^a differences in vivo, 3–6 × 10^6 unseparated T cells were transferred intravenously to Mls^a- vs. H-2-different mice exposed to 900 rad. T blasts were recovered from thoracic duct lymph (TDL) of the recipients at 3–5 d post-transfer, and typed for CD4, CD8, and Vβ expression. For Vβ8 expression, cells were typed separately for Vβ8.1 and Vβ8.2. Two experiments are shown in Table III.

In Table III, Exp. *1*, transfer of B10.BR (Mls^b) T cells to irradiated Mls^a-disparate AKR/J mice generated enormous numbers of blast cells, i.e., ~7.0 × 10^7 cells/mouse over the collection period of 48 h. The ratio of CD4$^+$/CD8$^+$ blasts was ~4:1 (Table III, Exp. *1*, lines *1–3*). The CD4$^+$ and CD8$^+$ blasts both showed conspicuously high Vβ6 expression, i.e., ~75% for CD4$^+$ blasts and 65% for CD8$^+$ blasts. The blasts also showed a twofold enrichment for Vβ8.1$^+$ cells (relative to resting T cells; see lower portion of Table III); Vβ8.2$^+$ blasts, by contrast, were almost undetectable. With transfer of B10.BR T cells to H-2-different (B10.P) recipients (Table III, Exp. *1*, lines *4* and *5*), TDL blast cells showed little or no enrichment for Vβ6$^+$ or Vβ8.1$^+$ cells relative to the input T cells.

In Table III, Exp. *2*, B6 T cells were transferred to Mls^a-disparate D1.LP mice. It should be noted that, unlike the strain combinations considered above, B6 and D1.LP mice are both I-E$^-$. With this combination the vast majority of the TDL blasts were CD4$^+$ cells, only ~6% of the blasts being CD8$^+$. These few CD8$^+$ blasts, however, were largely Vβ6$^+$, i.e., 50–60% (Table III, Exp. 2, lines *1* and *2*). This compared with <10% Vβ6$^+$ blasts for CD8$^+$ blasts generated in the H-2-different B6 → B10.P combination (Table III, Exp. 2, line *3*). For both strain combinations the blasts showed only low expression of Vβ8.1, Vβ8.2, and Vβ11 (1–8%).

TABLE II

$V_\beta6$ Expression on CD8$^+$ Blast Cells Stimulated with Mls^a-positive Stimulators in Vitro

				Percent of blasts expressing:			Percent of CD4$^+$ blasts expressing:		Percent of CD8$^+$ blasts expressing:	
Exp.	Responders	Stimulators	Stimulus	Thy-1	CD4	CD8	$V_\beta6$	$V_\beta8.1 + 8.2$	$V_\beta6$	$V_\beta8.1 + 8.2$
1	B10.D2 T	DBA/2	Mls^{a,c}	97.3	70.6	26.7	54.7	28.6	46.4	16.9
	B10.D2 T	B10.P	H-2^p	98.0	32.7	65.3	18.6	21.1	6.9	12.4
	B10.D2 CD8$^+$	DBA/2	Mls^{a,c}	96.4	0.6	95.8	-	-	41.5	21.8
	B10.D2 CD8$^+$	BALB/c	Mls^c	88.9	0.7	88.2	-	-	5.3	13.0
	B10.D2 CD8$^+$	B10.P	H-2^p	98.2	0.1	98.1	-	-	12.2	12.2
2	B10.BR T	CBA/J	Mls^{a,c}	-	80.4	19.6	66.8	20.0	35.2	14.1
	B10.BR CD8$^+$	CBA/J	Mls^{a,c}	-	0.4	98.7	-	-	62.7	20.2
	B10.D2 CD8$^+$	CBA/J	Mls^{a,c},H-2^k	-	0.2	99.1	-	-	25.9	26.1
3	B10.BR CD8$^+$	AKR/J	Mls^a	-	5.6	94.3	-	-	58.0	23.3
	B10.BR CD8$^+$	B10.P	H-2^p	-	0.4	98.1	-	-	15.3	18.2
	B10.D2 CD8$^+$	AKR/J	Mls^a,H-2^k	-	0.7	99.1	-	-	29.8	19.6
	B10.D2 CD8$^+$	B10.P	H-2^p	-	0.3	98.2	-	-	14.8	19.9
Normal unprimed LN T cells										
	B10.D2			98.6	62.9	35.7	8.6	15.9	12.9	31.6
	DBA/2			-	71.4	28.6	0.1	15.4	<0.1	19.7
	B10.BR			96.8	53.3	43.5	9.5	18.5	13.2	25.7
	AKR/J			-	72.0	28.0	<0.1	12.9	<0.1	14.9

Doses of 5 × 10^6 unseparated T cells or purified CD8$^+$ cells were cultured with 5 × 10^6 stimulator cells (mitomycin C-treated, T-depleted spleen cells) for 4 d. For CD8$^+$ responders, the cultures were supplemented with rIL-2 (5 U/ml). Blast cells were washed and stained for FACS analysis as described in Materials and Methods. The data for staining of control unprimed T cells refer to small cells rather than blast cells.

Discussion

The notion that T cell responses to Mls determinants involve only CD4$^+$ cells is widely held (1, 8), but appears to rest largely on the report of Janeway et al. (5) that Mls^a-stimulated T blasts are "primarily Lyt-1$^+$,23$^-$ cells." CD8$^+$ (Lyt-23$^+$) blasts were not undetectable in this study, however, and accounted for ~20% of the Thy-1$^+$ blasts. The data of Janeway et al. (5) are thus not incompatible with the present data, where 20–25% of the blast cells were CD8$^+$. The key question is whether these CD8$^+$ blasts are Mls specific or are induced nonspecifically. The present finding that exposure of CD8$^+$ cells to Mls^a-different stimulators selectively stimulates $V_\beta6^+$ blasts strongly suggests that stimulation of CD8$^+$ cells is indeed (Mls^a) specific.

TABLE III

Response of CD8$^+$ Cells to Mls^a Determinants In Vivo: $V_\beta6$ Expression on T Blasts Recovered from Thoracic Duct Lymph

							V_β expression on blasts							
					Percent of blasts expressing:		Percent of CD4$^+$ cells expressing:				Percent of CD8$^+$ cells expressing:			
Exp.	Cells injected	Irradiated hosts	Stimulus	Time of collecting TDL blasts	CD4	CD8	$V_\beta6$	$V_\beta8.1$	$V_\beta8.2$	$V_\beta11$	$V_\beta6$	$V_\beta8.1$	$V_\beta8.2$	$V_\beta11$
				h										
1	B10.BR T	AKR/J	Mls^a	72–87	83.6	16.7	75.6	21.8	<0.1	-	64.3	25.5	<0.1	-
				87–92	82.6	18.2	76.7	18.7	2.4	-	65.9	23.5	<0.1	-
				96–116	83.8	18.8	77.3	15.3	1.9	-	65.2	24.7	<0.1	-
	B10.BR T	B10.P	H-2^p	87–92	82.0	15.9	11.8	6.1	16.3	-	13.3	16.3	6.7	-
				96–116	74.1	29.3	12.8	5.3	16.6	-	16.1	12.9	10.0	-
2	B6 T	D1.LP	Mls^a	48–62	93.8	6.7	66.6	19.6	0.7	0.4	50.1	8.6	3.3	2.8
				72–84	94.6	5.6	70.6	14.9	0.7	0.2	60.2	7.8	1.5	0.9
	B6 T	B10.P	H-2^p	72–84	65.8	34.8	6.0	6.0	14.3	8.0	8.7	5.4	5.5	6.4
Normal unprimed LN T cells														
	B10.BR				53.3	43.5	9.5	3.1	15.4	0.1	13.2	11.3	12.4	1.1
	AKR/J				72.0	28.0	<0.1	<0.1	12.9	0.1	<0.1	0.4	14.5	0.1
	B6				50.5	48.5	8.6	6.4	12.1	4.4	6.6	7.6	8.8	6.1
	D1.LP				56.6	41.3	0.7	<0.1	15.0	5.4	0.9	2.6	10.9	7.1

Doses of 3–6 × 10^6 LN T cells were transferred intravenously to mice exposed to 900 rad 2 h before. Thoracic duct cannulas were inserted at 3 d post-transfer and lymph samples were collected over the intervals listed (10). Blast cells pooled from two to three mice/group were stained for FACS analysis as described in Materials and Methods.

Although nonspecific stimuli probably contribute to blast cells generated in vitro, blast cells stimulated in response to alloantigens in vivo are highly antigen specific. In this respect, it is notable that, of the $CD8^+$ blasts generated in vivo in the B10.BR → AKR/J combination, ~90% of the $CD8^+$ blasts expressed either Vβ6 (65%) or Vβ8.1 (25%). Since expression of other Mls^a-reactive Vβs, e.g., Vβ9 (12), was not examined, the data suggest that almost all $CD8^+$ blasts generated in the B10.BR → AKR/J combination are Mls^a specific (despite the numerous other antigenic differences between these two strains).

$V\beta6^+$ cells also accounted for the majority (up to 60%) of the $CD8^+$ blasts generated in the $I\text{-}E^-$ B6 → D1.LP combination. With this combination, however, the percent of $CD8^+$ blasts was quite low (<10%). Moreover, despite the preponderance of $V\beta6^+$ blasts, there was no enrichment for $V\beta8.1^+$ blasts. These data suggest that, in contrast to the other strains tested, the Mls^a reactivity of $CD8^+$ cells in the B6 → D1.LP combination is limited and applies only to $V\beta6^+$ cells and not to $V\beta8.1^+$ cells. Whether the different results obtained with the B6 → D1.LP combination are somehow related to lack of I-E expression in this combination is currently being explored.

Whether Mls^a-reactive $CD8^+$ cells are restricted by H-2 class I or class II molecules is still unclear. In considering this question, it is of interest that, like Mls^a antigens, class II alloantigens stimulate $CD8^+$ cells as well as $CD4^+$ cells (11). In two respects, the responses of T cells to Mls^a antigens and class II antigens exhibit close similarities. First, for each type of antigen, $CD4^+$ and $CD8^+$ cells show shared usage of particular Vβ TCRs, e.g., Vβ6 for anti-Mls^a response (this paper) and Vβ11 for anti-I-E responses (11). Second, in contrast to responses to class I antigens, responses of $CD8^+$ cells to Mls^a antigens or class II antigens only occur in the presence of $CD4^+$ cells or lymphokines (e.g., IL-2) released from these cells. In view of these similarities, we think it likely that anti-Mls^a responses by $CD8^+$ cells involve corecognition of class II molecules rather than class I molecules. This would explain why Mls^a-reactive $CD4^+$ and $CD8^+$ cells both tend to use the same Vβ TCR, e.g., Vβ6. To seek direct evidence on the H-2 class specificity of Mls^a-reactive $CD8^+$ cells, we are preparing cloned lines of these cells to test the blocking effects of anti-class I vs. anti-class II antibodies.

Summary

Contrary to existing dogma, evidence is presented that proliferative responses of mature unprimed T cells to Mls^a antigens involve $CD8^+$ cells as well as $CD4^+$ cells. The response of $CD8^+$ cells to Mls^a antigens proved to be heavily dependent on help from $CD4^+$ cells, and responses were stronger in three $I\text{-}E^+$ strain combinations than in an $I\text{-}E^-$ combination. In $I\text{-}E^+$ combinations, $CD8^+$ blast cells accounted for 20–25% of the blasts generated from unseparated T cells responding to Mls^a-bearing stimulator cells in vitro; similar findings applied to blast cells generated in vivo. The observation that the majority (≥50%) of Mls^a-stimulated $CD8^+$ cells (and $CD4^+$ cells) were $V\beta6^+$ indicated that $CD8^+$ cells respond to Mls^a antigens, per se, rather than to nonspecific stimuli. Whether $CD4^+$ and $CD8^+$ cells use the same or different H-2-restricting elements to respond to Mls^a antigens has yet to be resolved.

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