IN VITRO SENSITIZATION OF THYMOCYTES

Role of H-2 I Region Determinants and Cell-Free Mixed Leukocyte Culture Supernates in Generation of Cytotoxic Responses*

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In in vitro mixed leukocyte culture (MLC)[1] reactions, cytotoxic T lymphocytes (CTLs) are generated when responding and stimulating (usually γ-irradiated or mitomycin-C treated) cells differ for certain antigens determined by the major histocompatibility complex (1). Genetic experiments have revealed that in mice, the strongest of the antigens recognized by CTLs on the target cells are coded by K and D regions of the H-2 complex (2). Although there is sufficient evidence to indicate that differences between responding and stimulating cells of either the K or D region alone will lead to generation of CTLs, in most situations additional disparity for the H-2 I region is needed to obtain an optimal response. Such findings have led to the two signal hypothesis for in vivo and in vitro activation of CTLs (2, 3).

In recent years, several groups have demonstrated that soluble factors found in MLC supernates can, in the presence of a K- or D-region difference, substitute for the I region different stimulating cells by enhancing a weak cytotoxic response to a strong one (3–7). These studies have used spleen or lymph node cells as responders for the generation of CTLs. Unfractionated thymocytes, on the other hand, are poor responders to allogeneic stimulation in MLC and cell-mediated lympholysis (CML) assays unless supplemented with lymph node or spleen cells (8–10). Recently, Simon and coworkers (11) have demonstrated that a supernatant preparation from human MLC can help in the generation of in vitro CML response of murine thymocytes to alloantigens. In this communication, we report that thymocytes, when stimulated with H-2 different allogeneic spleen cells, give a strong proliferative response without developing a significant CML response. Addition of cell-free supernatant factor (SF) to these cultures leads to a significant CML response. However, contrary to findings using spleen cells as responders (12), thymocytes do not respond (even minimally under conditions tested) to either K- or D-region differences alone, irrespective of the

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[1] *Abbreviations used in this paper*: CML, cell-mediated lympholysis; CTL, cytotoxic T lymphocytes; EHAA, Eagle's Hanks' amino acid; E/T, ratio of effectors to ^{51}Cr-labeled targets in CML assay; LU, lytic units; MLC, mixed leukocyte culture; MR, maximum release; NMS, normal mouse serum; SF, cell-free supernatant factor; SR, spontaneous release.

presence of SF, unless additionally stimulated with an I-region encoded antigenic difference.

Materials and Methods

Mice. All inbred strains of mice used in this study are bred and maintained in this laboratory. Throughout this work, 4 to 6-wk-old mice of both sexes were employed.

Generation of SF. Responding C57BL/6 (BL/6) spleen cells were treated with water for 5 s to lyse erythrocytes and reconstituted to normal osmolarity with 10X phosphate-buffered saline. Stimulating B10.S cells were similarly treated and subsequently UV irradiated (13). 20 Million responders were mixed with 20×10^6 UV-irradiated stimulators in 20 ml Eagle's Hanks' amino acid (EHAA) containing 50 μM 2-mercaptoethanol and 0.5% normal mouse serum (NMS) in Falcon flasks (Falcon 3013 BioQuest, BBL, & Falcon Products, Cockeysville, Md.). After 7 days of incubation at 37°C in 5% CO_2 atmosphere, the cultures were spun at 5,000 *g* for 15 min. Resulting supernates were filtered through 0.45-μm Millipore filter (Millipore Corp., Bedford, Mass.) and stored in aliquots at −80°C.

In Vitro Sensitization of Cells. 30 million responding thymocytes or spleen cells were cultured with appropriate numbers of γ-irradiated (2,000 rads) spleen-stimulating cells in 20 ml EHAA containing 50 μM 2-mercaptoethanol and 0.5% NMS in Falcon flasks (Falcon 3013). The cultures were incubated and tested for MLC and CML.

Assays. MLC and CML assays are performed after 5 days of in vitro culture (unless given otherwise) according to Peck and Bach (14). Briefly for MLC, the cells were dispersed from the bottom of the flasks. 0.2 ml of this suspension were transferred to microtiter plate (Linbro IS-MRC-96-TC, Linbro Chemical Co., Hamden, Conn.) wells to which 2.0 μCi of [^{3}H]TdR were added. Plates were harvested after 6 h and results expressed as counts per minute of [^{3}H]TdR incorporated; standard deviations are included. For CML estimation, given number of viable effector cells were incubated with 1×10^4 ^{51}Cr-labeled phytohemagglutinin-stimulated lymph node target cells in 0.2 ml of EHAA with 5% fetal calf serum in round bottom microtiter wells (Linbro IS-MRC-96-TC). The plates were incubated for 3 h and the radioactivity released into the supernate was recovered using Skatron Titertek Supernatant Collection System (Flow Laboratories, Inc., Rockville, Md.). Spontaneous release (SR) represents the counts per minute released by 1×10^4 target cells and maximum release (MR) is that released by 1×10^4 detergent lysed target cells. Results are expressed as percent cytotoxicity calculated as:

$$\frac{(\text{cpm experimental release} - \text{cpm SR})}{(\text{cpm MR} - \text{cpm SR})} \times 100$$

or as lytic units (LU) per 1×10^6 effector cells, where 1 LU is the number of effector cells needed to achieve 33.3% lysis of ^{51}Cr-labeled target cells.

Results

MLC and CML Response of Thymocytes in the Presence of SF. Table I shows that when thymocytes from B10.A animals were stimulated with C57BL/6 spleen cells in the absence of SF, a strong MLC response was obtained. Under these conditions however, only a slight, if any, CML response was detected. On the other hand under identical conditions, with control spleen cell responding cultures, both a strong MLC as well as a strong CML response could be observed. Addition of SF[2] to a final concentration of 5% to the cultures containing thymocytes as responding cells, resulted in a strong CML response. CTLs generated under these conditions were specific in killing targets having the H-2 haplotype of the sensitizing cell (H-2^b). Thus, with responding thymocytes, stimulation with allogeneic cells differing by an entire H-2 haplotype in the presence of SF, results in the generation of CTLs that are specific for the sensitizing alloantigen.

[2] Optimal concentration of SF in cultures was separately determined and found to be 5% with thymus-responding cells and 20–25% with spleen cells as responders.

TABLE I

Response of Thymocytes and Spleen Cells to H-2 Disparate Stimuli

B10.A Responding cells	Stimulating spleen cells	SF	MLC Response [^{3}H]thymidine incorporated	CML ± SD Targets		
				B10.A	B10.G	BL/6
			cpm		%	
Thymocytes						
	B10.A	−	565 ± 172	2.9 ± 5.2	−	5.3 ± 4.2
	B10.A	+	613 ± 199	−1.0 ± 3.3	−	2.9 ± 2.1
	C57BL/6	−	7,846 ± 846	4.6 ± 5.8	6.6 ± 3.4	7.8 ± 1.8
	C57BL/6	+	9,308 ± 1,205	3.5 ± 0.9	7.6 ± 3.8	41.1 ± 3.7
Spleen						
	B10.A	−	873 ± 259	2.0 ± 4.8	−	4.8 ± 3.5
	B10.A	+	1,107 ± 212	−3.6 ± 3.1	5.6 ± 4.5	3.4 ± 5.1
	C57BL/6	−	17,839 ± 1,866	4.9 ± 2.8	8.7 ± 3.6	48.2 ± 1.6
	C57BL/6	+	11,335 ± 1,522	5.5 ± 2.6	8.2 ± 5.3	53.8 ± 2.4

Ratio of effectors to ^{51}Cr-labeled targets in CML assay (E/T) 40:1.
SF concentration as indicated in Table II.

Does SF Provide Help for Generation of CTLs in the Presence of K/D Region Stimulation? Many previous reports have indicated that cell-free supernatant preparation can substitute for T-helper cell function in in vitro humoral antibody (15) and cytotoxic allograft responses (3–7, 11). In the present experiments, using thymocytes as responding cells, we tested whether T-helper cell function for CTL development could be replaced with SF preparation. Experiments were designed to see if SF could substitute for an H-2 I-different stimulating cell in the presence of K or D region different stimulating cells alone. The results of such an experiment are described in Table II. When responding B10.A thymocytes were stimulated with cells from either B10.A(1R) (H-2 D difference) or AQR (H-2 K difference), no detectable CML response could be generated. Addition of SF to these cultures did not alter the results. However, in the control experiment using spleen cells as responders, a moderate but highly significant CML response is obtained against D- or K-region different stimulating cells which can be further enhanced by the addition of SF.

The inability of thymocytes to respond in CML to stimulation with H-2 K or H-2 D differences could not be changed by varying the dose of stimulating cells. As shown in Fig. 1 under the culture conditions employed, AQR thymocytes in the presence of 5% or 20% SF led to a significant CML response when stimulated with 2×10^6 C57BL/6 spleen cells (entire H-2 difference), whereas under identical conditions, stimulation with B10.A (H-2 K difference) spleen cells over a wide range ($2–50 \times 10^6$ cells) did not result in any detectable CML response. In control experiments with AQR spleen cells as the responding cells, a significant CML was obtained throughout the range of B10.A-stimulating cell concentrations tested. This response could be further augmented by the addition of 20% SF. These experiments suggest that the failure of thymocytes to respond to a H-2 K or D difference alone is not due to a differential antigen concentration requirement as compared with spleen cells.

TABLE II

Response of Thymocytes and Spleen Cells to Differences for K and D Regions or the Entire H-2 Complex

B10.A Responding cells	Stimulating spleen cells	H-2 Difference	CML* ± SD	
			−SF	+SF‡
			%	
Thymocytes				
	B10.A	−	−2.3 ± 4.9	−1.6 ± 3.1
	B10.A(1R)	*D*	0.6 ± 2.8	−2.0 ± 1.8
	AQR	*K*	−1.7 ± 6.2	1.3 ± 3.8
	C57BL/6	*K, I, S, D*	4.2 ± 2.9	33.7 ± 4.9
Spleen				
	B10.A	−	3.3 ± 5.6	1.9 ± 5.1
	B10.A(1R)	*D*	24.7 ± 3.5	63.6 ± 0.9
	AQR	*K*	27.3 ± 6.2	54.9 ± 4.3
	C57BL/6	*K, I, S, D*	68.0 ± 7.5	69.8 ± 3.8

*E/T 40:1 on target cells syngeneic to stimulator.

‡ SF concentration with thymocytes as the responding cells was 5%, whereas in spleen cells it was added to a final concentration of 20%.

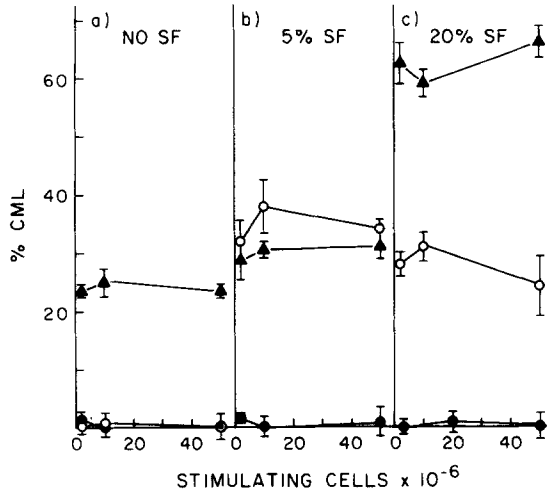

FIG. 1. Response of AQR cells to H-2 differences. 30 × 10^6 AQR thymus cells were stimulated with increasing numbers of B10.A (●) or C57BL/6 (○) cells in the absence or presence of SF; and compared with the response of AQR spleen cells stimulated with B10.A cells (▲). CML tested at an effector to target cell ratio of 40:1.

Another possible explanation for the apparent nonresponsiveness of thymocytes to K/D difference(s) alone, would be that activation was kinetically a slower process in this system; thus the CML response could require a longer period of time to be detectable. To test this, AQR thymocytes were stimulated with spleen cells differing for various regions of H-2 and the CML assayed on days 5 and 7. The results presented in Table III show that in cultures stimulated with a K-region difference

TABLE III

AQR Thymocyte Response to Various H-2 Differences

Stimulating Cells	H-2 Difference	Day 5 CML ± SD		Day 7 CML ± SD	
		−SF	+SF	−SF	+SF
		%		%	
AQR	−	−1.9 ± 3.4	2.5 ± 4.9	0.6 ± 4.3	1.5 ± 0.9
B10.A	*K*	3.1 ± 6.6	−1.2 ± 1.8	2.9 ± 6.3	−0.3 ± 2.7
B10.T(6R)	*I, S*	2.6 ± 1.5	4.6 ± 3.6	4.4 ± 2.6	3.5 ± 5.1
B10.A + B10.T(6R)	*K, I, S*	−0.2 ± 3.6	10.8 ± 2.3	−	8.7 ± 4.4
C57BL/6*	*K, I, S, D*	5.6 ± 4.4	36.4 ± 2.8	0.0 ± 3.4	23.4 ± 4.7

E/T 40:1.

Effectors were tested on B10.A targets, except * where C57BL/6 cells were used as targets.

alone essentially no difference is observed whether the CML is tested on day 5 or 7. In some experiments (data not shown), the cultures, even when allowed to go for an additional 2 or 3 days, did not generate any significant CML response when stimulated with K/D difference alone. Thus, lack of response to K/D by thymocytes appears not to be due to a delayed response.

Role of H-2 I Stimulation in CTL Activation in Thymocytes. It is evident from the preceding data that with thymocytes as the responding cells, I-region stimulation appears obligatory for CTL development under these conditions. Although SF can markedly enhance the development of a thymocyte cytotoxic response when the stimulating cells present K/D- and I-region encoded differences to the responding cells, SF appears to have no effect when only K/D region encoded differences are seen by the responding cells. These findings are, of course, in contrast to those seen with spleen cells as responders.

As has been previously reported (16), I-region and K/D-region stimulation can be effectively presented to spleen-responding cells on separate stimulating cells (three cell protocol). To investigate whether a similar situation exists with thymocytes as responding cells, AQR thymocytes were stimulated with B10.A spleen cells, and increasing numbers of B10.T(6R) (I + S difference) cells in the presence or absence of SF. As shown in Fig. 2, with spleen-responding cells, the addition of 2×10^6 B10.T(6R) cells strongly enhanced the CML response. The identical three cell protocol in the thymocyte system, even in the presence of SF and with up to 50×10^6 B10.T(6R) cells, resulted, at best, in only a weak CML response. In addition, the relatively weak response of thymocytes in the three cell experiment could not be attributed to a delay of the time of the peak response because as shown in Table III, the CML results obtained on day 7 were not significantly different from those obtained on day 5. These results suggest that for an optimal CML response from thymocytes, in addition to SF, K/D- and I-region differences should be provided on the same stimulating cell.

Is Nonresponsiveness of Thymocytes to K/D a Quantitative Phenomenon? It is possible that thymocytes do react to K/D difference alone, but the response is so weak that it cannot be detected by our assay system. Obviously, due to technical reasons, this

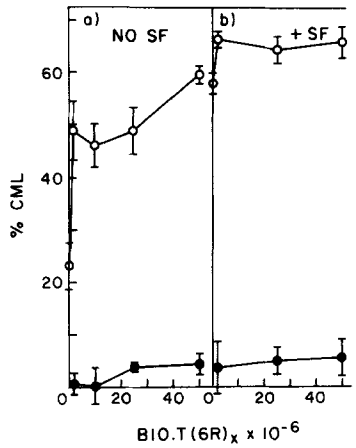

FIG. 2. Response of AQR thymus (●) or spleen (○) cells to B10.A, as a function of increasing numbers of B10.T(6R) cells. 30 × 10^6 AQR thymocytes or spleen cells were stimulated with 15 × 10^6 B10.A cells and indicated numbers of B10.T(6R) cells. SF concentration was 5% when added to thymocytes and 20% when added to spleen cells. Effector to target ratio was 40:1.

TABLE IV

Comparison of Two Cell and Three Cell Protocols

AQR Responders	Stimulating spleen cell	% CML ± SD Effectors per target*			LU/1 × 10^6 cells
		50	*12.5*	*3.1*	
Thymus					
	B10.A + SF‡	0.9 ± 4.5	3.6 ± 7.2	1.0 ± 4.1	0.00
	B10.A + B10.T(6R)	13.6 ± 4.2	5.1 ± 3.5	0.3 ± 3.0	0.08
	+ SF	16.0 ± 3.3	12.5 ± 3.8	6.7 ± 2.9	0.14
	C57BL/6	64.0 ± 5.0	43.3 ± 3.7	40.4 ± 2.5	16.81
	C57BL/6 + SF				
Spleen					
	B10.A	34.8 ± 3.9	18.4 ± 4.4	10.7 ± 3.1	2.26
	B10.A + SF	62.9 ± 4.2	47.5 ± 3.2	27.2 ± 2.6	21.21
	B10.A + B10.T(6R)	58.0 ± 6.9	44.1 ± 7.3	24.5 ± 3.7	18.12
	C57BL/6	67.8 ± 3.8	60.5 ± 4.5	38.3 ± 4.4	44.06

* C57BL/6 was used as the target cells in those groups that were stimulated with C57BL/6, in all others B10.A was used as the target cell.

‡ SF concentrations as shown in Table II.

point cannot be ruled out. However, it is possible to compare the magnitude of response under optimal conditions, i.e., in the presence of SF, between K/D and K/D + I stimulation and ascertain the minimum difference between the levels of responses to these stimuli. Such a comparison is presented in Table IV, where results have been also expressed in terms of LU/1 × 10^6 effector cells. When the number of lytic units generated in AQR thymocytes stimulated with B10.A in the presence of SF (line 1) is compared with that against C57BL/6 (line 4), it is evident that the response against

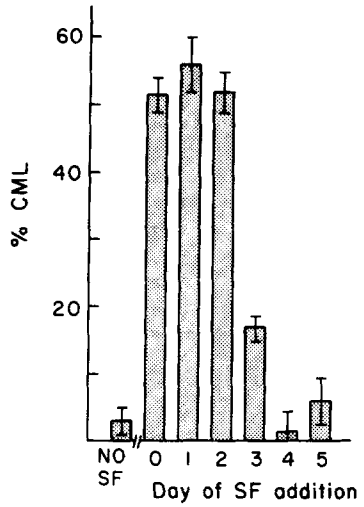

FIG. 3. Effect of time of addition of SF on the response of thymocytes. B10.A thymocytes were stimulated with C57BL/6 cells and SF added at the indicated time to a final concentration of 5%. Effector to target ratio was 50:1.

B10.A, if any, cannot be detected. Because, under these conditions even 0.05 LU/1 $\times$ 10^6 effector cells is a significant CML response, this thymocyte response to an H-2 K difference is at least 200-fold lower than against an entire H-2 difference. Under identical conditions, response of AQR spleen cells to K region difference alone was only $\cong$ 20-fold lower than against an entire H-2 difference. This difference was further reduced to only $\cong$ twofold upon the addition of SF, although different target antigens are involved in this comparison.

The Effect of Time of Addition of SF on the Response of Thymocytes to Allogeneic Stimulation. Fig. 3 shows the effect of time of addition of SF to the cultures on the response of B10.A thymocytes stimulated with C57BL/6 spleen cells; optimal restoration of CML response follows the addition of SF within 48 h of the culture initiation. The inability of the SF to activate a CTL response beyond this period could not be attributed to a delay in the course of events, because results obtained on day 8 of culture (data not shown) were essentially the same as those on day 5 as presented in Fig. 3.

Discussion

The studies described herein show that, under the conditions employed, thymocytes stimulated with H-2-different stimulating cells show a significant proliferative response, but fail to generate a significant cell-mediated cytotoxic response. Addition of SF to these cultures significantly improves the CML response, which is directed specifically against the sensitizing H-2 haplotype. There are, however, two surprising findings when the cytotoxic response of thymocytes is compared with that of spleen cells. First, with spleen as responding cells, as reported elsewhere (3), stimulation with H-2 differences localized to the K or D region alone, leads to a moderate but highly significant CML response, which can be enhanced by addition of I-region different stimulating cells or addition of SF preparations. However, with thymocytes as responding cells under these conditions, stimulation with K/D-region different spleen

cells both in the presence or absence of SF was ineffective in the activation of a CTL response. Second, although with spleen cells as a responding population, an enhancing influence of H-2 I-region different stimulating cells is detected even when K/D- and I-region differences are presented on separate stimulating-cell populations (the three cell protocol), with thymocyte-responding cells the three cell protocol, even in the presence of SF, engenders, at best, a very weak response.

Thus, in contrast to results obtained with spleen cells, in vitro CTL activation in thymocytes seems dependent not only on the presence of an H-2 K or D difference, but also on an H-2 I-region difference, which can be enhanced but not replaced with SF preparations. Furthermore, a three cell protocol with thymocytes as responding cells is a very inefficient stimulatory system for the activation of CTLs. A significant CML response is obtained only in the presence of SF and only when H-2 disparity between the responding thymocytes and the stimulating spleen cell includes both K/D- and I-region differences on the same stimulating cell.

The results summarized above may indicate a basic difference between thymocytes and the spleen cells in the response to an allogeneic stimulus. It is well established that a majority of thymocytes (>90%) are Ly-$1^+2^+3^+$, whereas a good proportion of peripheral T cells express the Ly-$1^+2^-3^-$ and Ly-$1^-2^+3^+$ phenotypes separately (17). Experiments using Ly antisera in MLC combinations differing by an entire H-2 complex have suggested that T cells of the Ly-$1^-2^+3^+$ phenotype proliferate and differentiate in response to H-2 K- and D-region determinants and generate cytotoxic killer cells, whereas Ly-$1^+2^-3^-$ cells respond to I-region determinants and provide T-helper cell function (17, 18). These and other experiments have led to the postulation of a two signal hypothesis for the activation of cytotoxic T-cells (2), in which a K/D-region difference provides signal 1 and an I-region difference stimulates Ly-$1^+2^-3^-$ helper T cells which provide signal 2 to the CTL for generation of a cytotoxic response.

Because the predominant population in thymocytes is an Ly-$1^+2^+3^+$ T cell, it is possible that, in contrast to the situation in peripheral T cells, in thymocytes both signals are received by the same cell. If such a situation exists, presentation of two signals on two different stimulating cells (three cell protocol) might be less effective as a result of competition and steric hindrance between two stimulating cells for the same precursor CTL. This suggestion however does not exclude the possibility of functional heterogeneity of responding thymocytes.

It is possible that the thymocyte response to K/D differences alone may not reflect any fundamental difference between the response of thymocytes as compared with that of spleen cells, but merely a difference in degree of response which is so weak that it is beyond the sensitivity of our methods of detection. If this is the case, our calculations (based on LU in Table IV) suggest that the reactivity of AQR thymocytes to B10.A cells (K-region difference) would be at least 200-fold lower than the CML response of AQR thymocytes to C57BL/6 cells (entire H-2 difference). Alternatively in the thymocyte system, absence of CTL response to a K/D difference alone, may result from the generation of suppression. Development of suppressor cells in response to HLA antigens (in the absence of functional help), has been previously demonstrated (19). The role of I-region stimulation in part may be to alleviate this suppression.

At this stage, we do not fully understand the role of SF in the generation of thymocyte CTLs. Under the present experimental conditions, it appears that addition

of SF to thymocyte cultures in the presence of K/D- plus I-region stimulation, leads to a significant CTL response, but unlike the situation with spleen responding cells, SF cannot substitute for the I-region stimulatory function in thymocytes. Because optimal levels of CTL response are only obtained if the factor is added within 48 h of the initiation of cultures, it seems likely that the factor(s) may at some level influence the differentiation of precursor CTLs or CTLs in very early stages after antigenic recognition. These studies further support the validity of a two-signal hypothesis for the activation of CTLs, and describe a stringent dependence of thymocyte activation on the presence of both signals.

Summary

Stimulation of thymocytes in vitro by spleen cells differing for the entire H-2 complex leads to a significant proliferative response without a significant cell-mediated lympholysis (CML) response. Addition of soluble cell-free supernates (SF), (taken from a 7-day mixed leukocyte culture) enables these cultures to develop CML response. For optimal CML response, the SF has to be added within 48 h of onset of cultures. Although with spleen cells as responding cells, SF could quantitatively replace I-region different stimulating cells for generation of CML responses, with thymocytes as responding cells, stimulation with I-region cells appeared obligatory for the generation of CML responses. The implications of these findings are discussed.

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