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THE DIFFERENTIATION OF CYTOTOXIC T CELLS *IN VITRO*

II. Amplifying Factor(s) Produced in Primary Mixed Lymphocyte Cultures Against K/D Stimuli Require the Presence of Lyt 2+ Cells but not Lyt 1+ Cells[1]

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Mixed lymphocyte cultures (MLC) contain soluble mediator(s) that are able to support primary cytotoxic responses to ultraviolet- (UV) inactivated allogeneic cells. The production of such amplifying factor(s) *in vitro* was studied by using spleen cells from a variety of B10 congenic mice, with a view to defining the antigenic and cellular requirements for mediator production. K, D, or I region stimulation in MLC all led to the production of amplifying factor(s). The kinetics of production of mediator after K/D region stimulation was not different from that observed with an I region stimulus. Furthermore, UV treatment of stimulator cells bearing K, D, or I region products led to a reduction in the ability of these cells to induce a primary cytotoxic response and to "trigger" the production of amplifying factor(s). Thus, in B10 congenic mice, K/D and I region encoded alloantigens were indistinguishable in their ability to induce amplifying factor(s) in primary MLC, in their susceptibility to UV irradiation, and in their ability to trigger the differentiation of cytotoxic T cells.

On the other hand, striking differences were noted in the cellular requirements for the production of amplifying factor(s) when K/D and I region stimulation were compared. Thus, I region stimulation required the presence of Lyt 1+ T cells (but not Lyt 2+ T cells) in order for mediator(s) to be produced. In contrast, factors produced in response to K/D region stimulation (with I region compatibility) were independent of Lyt 1+ cells, but required the presence of Lyt 2+ cells.

These findings suggest that amplifying factors may play a central role in the differentiation of cytotoxic T cells regardless of the alloantigenic stimulus. Furthermore, they provide additional evidence that the Ly phenotype of "helper" cells is influenced by the region of the MHC that is recognized.

Recent studies have indicated that the differentiation of cytotoxic T cells *in vitro* is associated with collaboration between T cell subpopulations (1–4). Such investigations have indicated that T cell-derived soluble mediators, which have been termed amplifying factors, may play an important role in the differentiation of cytotoxic cells (5–10). For example, stimulator spleen cells exposed to ultraviolet (UV) light are incapable of inducing a primary cytotoxic response *in vitro*, unless amplifying factors, in the form of cell-free supernatants from a mixed lymphocyte culture (MLC), are also added. We, and others, have shown that the production of such augmenting factors is dependent upon the presence of Lyt 1+ T cells, presumably conventional "helper" T cells, but is independent of Lyt 2+ T cells (8, 10). The effector cells that differentiate in response to the combined stimulus of UV-treated cells and augmenting factors have been shown to bear Lyt 2 alloantigens, but to lack Lyt 1 (10). These studies are thus in keeping with the general hypothesis, first advanced by Bach and his colleagues (3), that the proliferation of Lyt 1+ T cells in MLC leads to the production of mediators that may amplify the clonal expansion of Lyt 2+ cytotoxic precursor cells.

Recent studies involving recombinant and H-2 mutant mice, however, have given results that seem to be discordant with this model. These investigations suggest that the nature of the stimulating H-2 alloantigen complex may determine the subset of T cells that respond (11–13). Thus, in MLC in which responder and stimulator cell differ only at the K/D ends of the H-2 complex, it was noted that the rapidly proliferating cell (13) and the cytotoxic effector cell were both of the Lyt 1+2+ phenotype (11, 12). In these cases, it was not clear whether T-T collaboration occurred, nor whether soluble mediators played a role in the differentiation of the cytotoxic cells observed.

The studies presented here represent an attempt to answer two general questions: i) Do amplifying factors play a role in primary cytotoxic responses regardless of the antigenic stimulus (K, D, or I region stimulation) and ii) Is collaboration between the Lyt 1+ and Lyt 2+ T cell subsets a general feature of the primary cytotoxic response? Using B.10 congenic mice that differed in discrete regions of the H-2 gene complex, we observed that K, D, and I region stimulation in MLC all led to the production of amplifying factors. Interestingly, the cellular requirements for factor production differed with the stimulus. Thus, the production of amplifying factor(s) after I region stimulation required Lyt 1+ T cells, but not Lyt 2+ T cells. In contrast, factor production involving K/D region stimulation needed Lyt 2+ T cells, but was independent of the presence of cells bearing Lyt 1+ alloantigens. These findings imply that amplifying factors play a role in the differentiation of cytotoxic T cells after K/D stimulation. It seems likely that such factors are produced by Lyt 2+ cells.

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MATERIALS AND METHODS

Mice. Male C57BL/6, CBA/J and BALB/c mice (8 to 10 weeks old) were obtained from the Jackson Laboratories, Bar Harbor, Maine. Male B10.AQR, B10.A, B10.A(2R), B10.A(5R), and B10.T(6R) mice were obtained from the central animal facility of the Fred Hutchinson Cancer Research Center.

Tumor cells. The mouse mastocytoma P815-Y (DBA/2 origin) and EL-4 cells (C57BL/6 origin) were maintained as ascites tumors by weekly passage in syngeneic mice. Cells were harvested by peritoneal aspiration.

Reagents and antiserum. 2-Mercaptoethanol (2-ME),[2] and mitomycin C (Mit-C) were purchased from the Sigma Chemical Company (St. Louis, Mo.). The anti-Thy-1.2 (anti-θ) serum used was raised in AKR mice against C3H thymus cells. The specificity of the antiserum has been previously described in detail (10).

Anti-Lyt 1.2 sera were prepared essentially as described by Shen *et al.* (14). C3H/HeJ mice were injected with thymus cells from C3H.CE (Ly 1.2) mice. Recipient mice were immunized subcutaneously with two thymi per 10 animals, followed by five progressively increasing i.p. doses (about one thymus/one mouse) at monthly intervals. This anti-Lyt 1.2 sera had a titer of more than 1:400 on Ly 1.2 thymus cells and no detectable titer on C3H/HeJ thymus cells. The anti-Lyt 2.2 serum was a gift from Dr. R. Nowinski, of the Fred Hutchinson Cancer Research Center, Seattle, Washington and was raised by the method of Shiku *et al.* (15). This anti-Ly 2.2 sera had a titer of 1:160 on Ly 2.2 thymus cells. The specificity of all antisera was ascertained by assessment of their ability to abrogate "helper" cells in PFC formation, as the anti-Lyt 1 serum did, or alloimmune cytotoxic T cells, observed with the anti-Lyt 2 serum. The anti-Lyt 2 serum had no effect on helper cells in PFC, the anti-Lyt 1 serum reduced T cell-mediated cytotoxicity against allogeneic spleen cells < 15%.

Treatment of spleen cells with the anti-Ly sera was carried out by using 10^7 spleen cells/ml and a 1/20 dilution of the antiserum. Treatment was carried out for 30 min at 37°C, after which time the cells were washed once, and then incubated with normal rabbit serum (1/10 dilution) for a further 45 min at 37°C as described previously (10).

Fraction of spleen cells on nylon wool. A T cell-enriched cell population was obtained by passage of spleen cells through a lightly packed glass wool and nylon wool column by using a procedure modified (16) from that described by Julius *et al.* (17).

Treatment of lymphoid cells with UV light. UV light irradiation of stimulator spleen cells was performed as previously described (10). Briefly, a 3-ml cell suspension (10^7/ml, in Hanks balanced salt solution, HBSS) was exposed in an open 60-mm Petri dish to 380 $\mu W/cm^2$ UV light for 10 min. After UV light treatment, the cells were washed and resuspended in RPMI 1640 culture medium containing 10% fetal calf serum (FCS, Grand Island Biological Co., Grand Island, N. Y.).

Induction of primary cytotoxic responses. Spleen cell suspensions, freed of erythrocytes, were prepared as previously described (18). Cultures were established in flat-bottomed Falcon Plates (3008 Falcon Plates; Oxnard, Calif.). The culture medium was RPMI 1640, enriched with 10% FCS and containing 100 units/ml of penicillin and 100 μg/ml of streptomycin. The FCS was preselected for its ability to support the production of amplifying factor(s) and the induction of cytotoxic T cells *in vitro*.

T cell-enriched spleen cells (5×10^6) obtained from normal C57BL/6 mice were cultured in RPMI 1640 containing 10% FCS and 5×10^{-5} M 2-ME. To these cultures, 5×10^5 mitomycin C- (Mit-C)-treated (50 μg/ml, 45 min, 37°C) BALB/c spleen cells, which had (or had not) been exposed to UV-treatment were added. Cell cultures (total volume 1.8 ml) were incubated for 5 days at 37°C in an atmosphere of 95% air, 5% CO_2. At the end of culture, cells were harvested, washed once with RPMI 1640 containing 10% FCS, and the viable cell content was assessed with an erythrocin B dye exclusion assay.

Induction of amplifying factor(s). 10^7 Normal B10.AQR spleen cells were cultured in RPMI 1640 + 10% FCS with 10^7 Mit-C treated (50 μg/ml, 45 min, 37°) B10.A, B10.A(2R), B10.A(5R), or B10.T(6R) spleen cells in flat-bottomed Linbro plates (Linbro Scientific, New Haven, Conn.). After 36 hr incubation at 37°C, cultures were harvested, centrifuged at 300 × G for 10 min, and the cell-free supernatants were recovered and used as a source of amplifying factor.

For the production of secondary MLC supernatants, 25×10^6 B10.AQR spleen cells were incubated with an equal number of irradiated (1400 rads) B10.A, B10.A(5R), or B10.T(6R) spleen cells in 20 ml medium in 25 cm^2-tissue flasks (3013 Falcon Plastics) kept in an upright position. 10^7 Viable cells were harvested from these primary cultures on day 14 and further cultured with 10^7 Mit-C treated B10.A, B10.A(5R), or B10.T(6R) spleen cells in flat bottomed Linbro plates. After 24 hr incubation at 37°C, cell-free supernatants were recovered and used as a source of "secondary" amplifying factor. All supernatants were centrifuged at 600 × G for 10 min and then dialyzed extensively, first against HBSS and then against RPMI 1640 + 10% FCS, before use.

Cytotoxic assay. The cytotoxic activity of spleen cell cultures was assayed by using a microcytotoxicity assay employing ^{51}Cr-labeled P815 target cells (19). Briefly, 50×10^4 effector cells were mixed with 10^4 ^{51}Cr-labeled target cells in conical well microtiter plates (Linbro Scientific), in a reaction volume of 0.2 ml RPMI 1640 containing 10% FCS. After centrifugation (30 sec, 400 × G), the cultures were incubated at 37°C for 4 hr. An aliquot of cell-free supernatant was then taken and its ^{51}Cr content was determined.

The cytolytic activity was determined as follows:

$$= \frac{\text{Experimental } ^{51}\text{Cr release} - \text{control } ^{51}\text{Cr release}}{^{51}\text{Cr released after three cycles of freeze-thaw}} \times 100$$

Control assays contained target cells incubated in the presence of RPMI 1640 + 10% FCS.

RESULTS

Characterization of specificity of congenic mice. To ensure that the B10 congenic mice employed in this study were of the postulated genetic composition (Table I), specificity tests were carried out by measuring the development of cytotoxic cells in MLC. As can be seen in Table II, MLC between combinations differing in the K region of H-2 (B10.AQR + B10.A),[3] or in both K and D regions (B10.AQR + B10.A(2R)), generated significant cell-mediated cytotoxicity against appropriate target cells. Rel-

[2] Abbreviations used in this paper: Mit-C, mitomycin C; 2-ME, 2-mercaptoethanol; HBSS, Hanks' balanced salt solution; CTL, cytotoxic T lymphocytes.

[3] It seems unlikely that the recently postulated I-N region (20) would confuse our contention that the B10.A, B10.AQR combination represents only a K region mismatch. Thus, anti I-N^k antisera which lyse B10.BR cells also lyse cells from B10.AQR mice suggesting they share putative I-N specificites (20). As B10.BR and B10.A share I-N^k specificity, it seems likely that B10.AQR is also of this phenotype.

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atively weak cytotoxicity was generated when I region stimulation was primarily involved (e.g., in cultures of B10.AQR and B10.T(6R) cells). These results were in keeping with several other reports (21-23). The specificity of cytotoxicity developing in each case (Table II) was in concordance with the presumed genetic constitution of the mice (Table I), but there was some evidence of preference for the stimulator cell. Thus, in each of several experiments of this type, B10.A cells were more effectively lysed than were B10.A(2R) when B10.A had been the stimulator cell, despite the fact that the cytotoxic cells were presumably anti-K^k, a specificity represented on both target cells.

Amplifying factor(s) production after K/D region stimulation. In order to investigate whether K/D region stimulation could produce amplifying factor(s) capable of augmenting primary cytotoxic responses, the following experiments were carried out. 10^7 Normal B10.AQR spleen cells were cultured with 10^7 B10.A$_{Mit}$ spleen cells (a K region stimulus) or with B10.A(2R)$_{Mit}$ spleen cells (K, D, and Tla stimulation). As positive controls, B10.T(6R)$_{Mit}$ spleen cells, and B10.A(5R)$_{Mit}$ spleen cells were also used to stimulate B10.AQR responder cells. Cell-free supernatants were harvested from all cultures 36 hr later. The presence of amplifying factor(s) in these supernatants was then assayed by culturing 5×10^6 normal C57BL/6 splenic T cells (nylon-wool passed splenocytes) for 5 days with 5×10^5 UV-irradiated BALB/c$_{Mit}$ spleen cells in the presence of varying concentrations of the supernatants. As we have previously reported (10), only minimal cytotoxicity developed in all cultures unless amplifying factor(s) were added, and little or no amplifying activity was seen in harvests from cultures containing B10.AQR + B10.AQR$_{Mit}$ or from cultures of B10.AQR spleen cells alone.

TABLE I

H-2 genotypes of mouse strains employed[a]

Strain	H-2 Haplotype	Origin of H-2 Region: K	I: A	I: B	I: J	I: E	I: C	S	D	Tla	Mls
B10.AQR	y1	q	k	k	k	k	d	d	d	a	b
B10.A	a	k	k	k	k	k	d	d	d	a	b
B10.A(2R)	h2	k	k	k	k	k	d	d	b	b	b
B10.A(5R)	i5	b	b	b	k	k	d	d	d	a	
B10.T(6R)	y2	q	q	q	q	q	q	q	d	a	b

[a] Data compiled from (29).

As can be seen in Table III, significant amplifying activity was observed in culture supernatants harvested from cultures involving K or K/D stimulation even in the absence of an I region stimulus. This was true for a variety of supernatant concentrations (range 5 to 60%). Moreover, in data not shown, amplifying activity was observed in culture supernatants harvested from cultures containing B10.A + B10.A(2R)$_{Mit}$ (D, Tla stimulus). The MLC supernatants did not induce cytotoxicity in the absence of stimulator cells (Table III). Furthermore, the cytotoxicity that developed after culture with UV-irradiated stimulator cells in the presence of MLC supernatants was specific for the priming alloantigen. By employing spleen cell "blasts" as targets, this was found to be true not only for supernatants harvested after K/D region stimulation, but also after I region stimulation (data not shown; see also (10)). No relationship was seen between the specificity of the cytotoxicity generated and the stimulus used to generate the amplifying MLC supernatant. In all cases, cytotoxicity was eliminated by treatment with anti-Thy 1.2 serum in the presence of rabbit complement (C). There was no indication that, under the conditions employed, K or D region stimulation was less effective at inducing factor production than was I region (or K + I region) stimulation.

Kinetics of amplifying factor production after K/D region stimulation. To investigate the kinetics of amplifying factor production, 10^7 B10.AQR spleen cells were mixed with 10^7 Mit-C-treated B10.A, B10.A(5R), or B10.T(6R) spleen cells, and cultured supernatants were harvested at frequent intervals. As previously, the amplifying effects of these supernatants were assayed by addition to cultures containing C57BL/6 T responder cells and UV-irradiated BALB/c stimulator cells. All supernatants were tested for amplifying activity at a variety of concentrations (range 5 to 60%). Maximal activity was noted in all cases at concentrations of 25 to 50% (v/v). As can be seen in Figure 1, regardless of the stimulus used, augmenting factor(s) were detectable within 24 hr of culture initiation, and maximal activity in all culture supernatants was noted by 48 hr. In other experiments, with the use of different batches of supernatants, similar kinetics were observed. These results demonstrated that there was no significant difference between the kinetics of amplifying factor production in cultures stimulated by K region encoded alloantigens in the absence of an I region stimulus and those stimulated by I region encoded specificities.

Supernatants harvested from secondary MLC have more amplifying activity than do supernatants from primary MLC.

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TABLE II

Primary cytotoxic responses in B.10 congenic mice[a]

Responder	Stimulator	H-2 stimulus	Expt.	B10.A I	B10.A II	B10.A(2R) I	B10.A(2R) II	B10.A(5R) I	B10.A(5R) II	B10.T(6R) I	B10.T(6R) II	B10.AQR I	B10.AQR II
B10.AQR	B10.A$_{Mit}$	K		83	40	20	24	3	0	3	−2	−4	−5
B10.AQR	B10.A(2R)$_{Mit}$	K, D, Tla		11	14	84	31	2	2	3	−1	−5	−4
B10.AQR	B10.A(5R)$_{Mit}$	K, I		9	4	19	7	89	57	16	1	−4	−2
B10.AQR	B10.T(6R)$_{Mit}$	I, S		6	2	10	2	10	3	37	13	−2	−2
B10.AQR	B10.AQR$_{Mit}$			−2	0	3	1	−2	0	11	0	−4	−2
B10.AQR				1	1	1	3	0	−1	3	2	−3	0

[a] 5×10^6 B10.AQR spleen cells were cultured with 10^6 B10.A$_{Mit}$, B10.A(2R)$_{Mit}$, B10.A(5R)$_{Mit}$, B10.T(6R)$_{Mit}$, or B10.AQR$_{Mit}$ spleen cells in the presence of 5×10^{-5} M 2-ME for 5 days. The cytotoxic activity of cells harvested from these cultures was assayed against LPS-blasts of B10.A, B10.A(2R), B10.A(5R), B10.T(6R), and B10.AQR spleen cells. The target cell blasts were prepared by incubating spleen cells with 10 μg/ml LPS for 2 days. The data shown is from a 4-hr cytotoxic assay. The data shown in both Expt. I and II was for effector cells to target cell ratio for 50: 1.

TABLE III

K/D region stimulation can lead to production of augmenting factor(s)[a]

Responder	Stimulator	Addition of Supernatants from:	(% v/v)	H-2 Difference	% Specific Cytolysis		
					Expt. I		Expt. II
					50:1	12:1	50:1
			%				
BL/6 T	BALB/c_{Mit}	None			91	78	90
	U.V.BALB/c_{Mit}	None			27	4	16
		B10.AQR + B10.A(2R)$_{Mit}$	5	K, D, Tla	65	27	N.D.[b]
			10		67	28	
			30		58	24	
			60		40	15	
		B10.AQR + B10.A(5R)$_{Mit}$	5	K, I	40	17	31
			10		74	37	48
			30		63	30	59
			60		58	25	45
		B10.AQR + B10.T(6R)$_{Mit}$	5	I, S	26	9	28
			10		40	16	38
			30		50	21	72
			60		51	26	74
		B10.AQR + B10.A_{Mit}	5	K	45	18	33
			10		36	14	56
			30		63	37	72
			60		36	16	74
		B10.AQR + B10.AQR_{Mit}	5	None	7	3	N.D.
			10		16	6	
			30		20	8	
			60		15	5	
		B10.AQR only	5		9	4	12
			10		14	3	12
			30		32	11	20
			60		30	11	20
Bl/6 T	None	None			0	0	1
		B10.AQR + B10.A(5R)$_{Mit}$	30	K, I	N.D.		0
			50				2
		B10.AQR + B10.T(6R)$_{Mit}$	30	I, S	N.D.		1
			50				1
		B10.AQR + B10.A_{Mit}	30	K	N.D.		0
			50				1
		B10.AQR only	30				1

[a] 10^7 Normal B10.AQR spleen cells were cultured with equal numbers of normal B10.A_{Mit}, B10.A(2R)$_{Mit}$, B10.A(5R)$_{Mit}$, B10.T(6R)$_{Mit}$, or B10.AQR_{Mit} spleen cells for 36 hr. Supernatants were harvested from these cultures and assayed for the presence of amplifying factor(s) by addition (at a final concentration of 5 to 60%) to cultures containing 5×10^6 C57BL/6 splenic T cells and 5×10^5 UV-irradiated (BALB/c)$_{Mit}$ spleen cells in the presence of 5×10^{-5} M 2-ME. All cultures were harvested after 5 days and their cytotoxic activity assessed against P815 target cells in a 4-hr assay. The two experiments shown employed different batches of supernatant. In each experiment, duplicate cultures were harvested for each of the culture conditions and the product pooled. The cytotoxicty assays were performed with duplicate aliquots at each effector to target cell ratio. The mean values, which has a S.E. of less than 10% in all cases, are given.

[b] N.D., not determined.

We have observed that cell populations containing "primed" helper T cells produced greater amounts of amplifying factor(s) than did populations of unprimed T cells.[4] Moreover, "primed" populations required less antigen to elicit factor production. Thus, alloantigen-primed cell populations were quantitatively distinguishable from unprimed populations. Wagner and Rollinghoff (8) have reported that primed helper T cells are able to produce amplifying factor(s) after restimulation with antigens encoded for solely by the I region of H-2. In order to investigate whether antigens encoded for by the K region could also prime

[4] Okada, M. and C. S. Henney. The differentiation of cytotoxic T cell *in vitro*. III. The role of helper T cells and their products in the differentiation of cytotoxic cells from "memory" cell populations. J. Immunol. In press.

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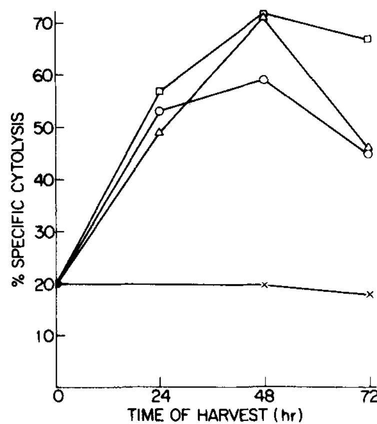

Figure 1. 10^7 B10.AQR spleen cells were cultured with 10^7 B10.A_{Mit} (□), with B10.A(5R)$_{Mit}$ (○), or with B10.T(6R)$_{Mit}$ (△) spleen cells. Additionally, 10^7 B10.AQR spleen cells were cultured alone (×). Cultures were established in duplicate and supernatants from all cultures were harvested at 24-hr intervals, pooled, and assayed. The presence of amplifying factor in supernatants was assayed in duplicate by addition to cultures containing 5×10^6 C57BL/6 splenic T cells and 5×10^5 UV-irradiated BALB/c_{Mit} spleen cells. Cultures were assayed for cytotoxic activity on day 5 by using P815 target cells in a 4-hr ^{51}Cr release assay at an effector cell to target cell ratio of 12:1. Mean values of duplicate assays are recorded. The data shown is for 30% (v/v) addition of cellfree supernatants. When other concentrations were used, similar kinetics were observed.

those T cells responsible for amplifying factor production, the following experiments were carried out.

B10.AQR spleen cells were cultured for 14 days with x-irradiated B10.A spleen cells. At the end of this period, 10^7 viable cells were harvested and restimulated with B10.A_{Mit} spleen cells. Supernatants were harvested after 24-hr culture and amplifying factor activity was assessed as previously. As can be seen in Figure 2, supernatants harvested from secondary MLC demonstrated greater amplifying activity than did comparable harvests from primary MLC. This was true regardless of the alloantigenic stimulus (Fig. 2) and at all supernatant concentrations tested (range 5 to 60%) (data not shown). To show that the alloantigen-primed populations were indeed mounting a 2° response and not simply a heightened primary both normal and B10.A-primed B10.AQR cells were stimulated with a third-party allogeneic stimulus (B10.T(6R) spleen cells). The amplifying activity induced under these circumstances was identical (data not shown).

These findings strongly suggest that antigens encoded for by the K and D regions, like I region products, are able to prime and to restimulate lymphoid cell populations to produce amplifying factor(s).

Effect of UV treatment on K/D region stimulation. We have previously reported (10) that UV-irradiated stimulator cells failed to induce either cytotoxic T lymphocytes (CTL) or amplifying factors during primary culture with allogeneic lymphocytes. In those studies, stimulation involved both K/D and I region encoded specificities and it was impossible to delineate which gene products were affected by the UV irradiation. It has been commonly assumed, however, that the activity of I region products is particularly susceptible to UV irradiation (24).

In order to study whether UV irradiation might affect the stimulatory capacity of antigens encoded for by the K/D regions of H-2, B10.AQR spleen cells were cultured with B10.A_{Mit}, B10.A(2R)$_{Mit}$, B10.A(5R)$_{Mit}$, or B10.T(6R)$_{Mit}$ spleen cells that had (or had not) been exposed to UV treatment. None of the UV-treated stimulator cells induced CTL formation. In order to examine whether UV-treated cells could induce amplifying factor(s), cultures were set up in an identical manner and cell-free supernatants harvested at 36 hr and assayed for the presence of amplifying factor(s) as previously.

As can be seen in Table IV, UV treatment of stimulator cells almost totally suppressed the ability of these cells to induce production of augmenting factor(s). These inhibitory effects were noted whether stimulation involved either K/D or I region specificities and were observed regardless of whether unfractionated spleen cells were used as stimulators. Thus, there was no evidence that I region encoded structures were more susceptible to the effects of UV irradiation than were those molecules coded for by the K/D regions of the H-2 complex.

Interestingly, UV-treated B10.A stimulator cells (unfractionated spleen cells or T cell-depleted splenocytes) cultured with a source of amplifying factor(s) readily induced a cytotoxic response in B10.AQR responder cell populations, although they did not do so in the absence of amplifying factor(s) (Table V). Moreover, supernatants harvested from B10.AQR + B10.A_{Mit} (K difference) were also able to restore cytotoxic responses in those cultures containing UV-treated stimulator cells (data not shown).

Production of amplifying factor(s) after K/D region stimulation is dependent upon Lyt 2+ cells. In order to investigate whether the cellular requirements for producing augmenting factor(s) after stimulation with K/D-encoded specificities were the same as those needed for responses to I region-encoded structures, the following experiments were carried out.

B10.AQR spleen cells were treated with anti-Lyt 1.2, anti-Lyt 2.2, or anti-Thy 1.2 serum antisera in the presence of C. Since mitomycin-treated spleen cell populations continued to produce amplifying factors (data not shown; see (10)), stimulator cells were treated with anti-Thy 1.2 serum and C to eliminate the possibility that stimulator cells might contribute to the production of amplifying factors. Consequently, T cell-depleted (anti

FACTOR PRODUCTION CONDITIONS — RELATIVE ACTIVITY PRODUCED (% Specific Cytolysis) 0 20 40 60 80

Responder	Stimulator	Stimulus	Response
B10.AQR	B10.A(5R)	K, I	1°
			2°
B10.AQR	B10.A	K	1°
			2°
B10.AQR	B10.T(6R)	I, S	1°
			2°
B10.AQR	B10.AQR		

Figure 2. 10^7 B10.AQR spleen cells were cultured with equal numbers of B10.A(5R)$_{Mit}$, B10.A_{Mit}, or B10.T(6R)$_{Mit}$ spleen cells. Culture supernatants were harvested at 24 hr (1° response). Additionally, 2.5×10^7 B10.AQR spleen cells were cultured with 2.5×10^7 B10.A(5R), B10.A, or B10.T(6R) x-irradiated spleen cells for 14 days *in vitro.* At the end of this period, 10^7 viable cells were recovered and restimulated with 10^7 cells of the phenotype used for priming. Cellfree supernatant was harvested 24 hr later (2° response). The presence of amplifying factor in all supernatants was assayed by addition of several concentrations of supernatants to cultures containing 5×10^6 C57BL/6 T cells and 5×10^5 UV-irradiated BALB/c spleen cells. Cultures were assayed for cytotoxic activity on day 5 by using ^{51}Cr P815 cells as targets and a 4-hr ^{51}Cr release assay. The data shown are for effector cell to target cell ratio of 50:1 and a supernatant concentration of (30% v/v). In all cases, this concentration of supernatant gave an optimal augmenting activity, but the 2° factors gave greater augmentation than did factors harvested from primary cultures at all concentrations tested (range 5 to 50%).

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TABLE IV

UV irradiation ablates the ability of both K/D and I region products to stimulate the production of amplifying factor(s)[a]

Responder	Stimulator	Addition of Supernatants from	H-2 Difference	% Specific Cytolysis					
				Expt. I		Expt. II		Expt. III	
				50:1	12:1	50:1	12:1	50:1	12:1
BL/6T	U.V.-BALB/c	None		3	−1	0	0	19	13
		B10.AQR + B10.A(5R)$_{Mit}$	K, I	27	8			56	39
		B10.AQR + UV-B10.A(5R)$_{Mit}$		8	2			36	20
		B10.AQR + B10.T(6R)$_{Mit}$	I, S	16	5	74	37	51	36
		B10.AQR + UV-B10.T(6R)$_{Mit}$		3	0	2	1	24	15
		B10.AQR + B10.A$_{Mit}$	K	17	4	31	12	59	34
		B10.AQR + UV-B10.A$_{Mit}$		6	1	0	0	28	17
		B10.AQR + B10.A(2R)$_{Mit}$	K, D, Tla					39	27
		B10.AQR + UV-B10.A(2R)$_{Mit}$						21	12
		B10.AQR only	None	5	0	0	0	12	6

[a] 10^7 Normal B10.AQR spleen cells were cultured with equal numbers of normal B10.A$_{Mit}$, B10.A(5R)$_{Mit}$, or B10.T(6R)$_{Mit}$ spleen cells that had (or had not) been exposed to UV irradiation. Supernatants from these MLC were harvested 36 hr later. The presence of amplifying factor in these cellfree harvests was assayed by culturing 5×10^6 C57BL/6 splenic T cells and 5×10^5 UV-irradiated BALB/c$_{Mit}$ spleen cells for 5 days in the presence of 30% (v/v) of cellfree supernatants. The resulting cultures were assayed for cytotoxic activity as described in Table III. The three experiments shown employed different batches of supernatants.

TABLE V

Primary cytotoxic responses of B10.AQR T cells to combined stimulus of UV-irradiated stimulator cells and MLC-derived amplifying factor(s)[a]

Cells Cultured		Supernatants Harvested from		% Specific Cytolysis ^{51}Cr-B10.A	
Responder	Stimulator			50:1	12:1
B10.AQR T cells	B10.A Spl$_{Mit}$	None		55	46
	U.V.B10.A Spl$_{Mit}$	None		4	4
	U.V.B10.A Spl$_{Mit}$	BL/6 + CBA/J$_{Mit}$	10%	11	8
			30	38	21
			50	52	35
	U.V.B10.A Spl$_{Mit}$	BL/6 only	10%	−1	3
			50	−1	0
	None	None		−1	0

[a] 5×10^6 B10.AQR splenic T cells were cultured with 5×10^5 UV-treated B10.A$_{Mit}$ spleen cells, in the presence (or absence) of supernatants harvested 36 hr after initiation of primary MLC between C57BL/6 and CBA/J$_{Mit}$ spleen cells. All cultures were harvested after 5 days and their cytotoxic activity assayed against B10.A (LPS-blast) target cells in a 4-hr assay.

Thy 1.2 treated) B10.A$_{Mit}$, B10.A(2R)$_{Mit}$, B10.A(5R)$_{Mit}$, or B10.T(6R)$_{Mit}$ spleen cells were used as stimulator cells. After 36 hr, cell-free supernatants were harvested from all cultures and the amplifying activity in the supernatants was assayed (as described in Table III). The experiments shown in Figure 3 were carried out at the same time by using the same responder cells.

As can be seen (Fig. 3), when B10.AQR spleen cells were treated with anti-Thy 1.2 serum and C and then cultured with T cell-depleted stimulator populations, no amplifying activity was produced in any of the cultures. This finding confirmed our previous demonstration (10), and the observations of others (5, 6), that amplifying factor production in primary MLC was T cell dependent.

B10.AQR spleen cells, depleted of Lyt 1.2 bearing cells, failed to produce mediator to either an I region (B10.T(6R)) or K plus I region (B10.A(5R)) stimulus. On the other hand, when the B10.AQR responder cells were treated with anti-Lyt 2.2 serum and C and then stimulated with the same alloantigens, marked amplifying activity was demonstrable in the harvested supernatants. It is thus clear, and in keeping with our previous report (10), that Lyt 1+ T cells are essential for the production of amplifying factor(s) in primary MLC when either I region, or K + I region stimulation is involved.

In contrast, as can be seen in the lower half of Figure 3, when the B10.AQR spleen cells were treated with anti-Lyt 2.2 serum and C and then stimulated with T cell-depleted B10.A$_{Mit}$ or B10.A(2R)$_{Mit}$ spleen cells, little amplifying activity was demonstrated. Thus, Lyt 2-depleted populations did not produce mediators to K/D region stimulation. However, B10.AQR spleen cells treated with anti-Lyt 1.2 serum and C readily produced amplifying factor(s) against these alloantigens (Fig. 3). This finding strongly suggests that Lyt 2+ T cells, but not Lyt 1+ T cells, are essential for the production of amplifying factors to K/D region stimulation.

DISCUSSION

The work described here extends the concept that soluble mediators play an important role in the differentiation of cytotoxic T cells *in vitro* (5–10). The studies specifically show that K/D and I region-encoded products can stimulate both CTL differentiation and the production of a factor(s) that can amplify cytotoxic responses to UV-inactivated stimulator cells. The kinetics of mediator production after K/D region stimulation were similar to that involving I region stimulation (Fig. 1). Thus, K/D region-encoded alloantigens could not be distinguished from I region encoded specificities either in their ability to induce CTL formation, or in their ability to induce mediators that amplify cytotoxic responses. Similar findings were made with both primary and secondary cytotoxic responses (Fig. 2), suggesting that K/D region specificities, like I region products, can "prime" lymphoid cell populations for secondary CTL formation and for secondary mediator production. Interestingly, the stimulatory capacity of both K/D and I region specificities was ablated after UV irradiation (Table IV). Thus, although B10.AQR spleen cells produced mediator (Fig. 1) and CTL (Table II) when stimulated with untreated B10.A or B10.T(6R) spleen cells, neither mediator nor CTL were induced after the

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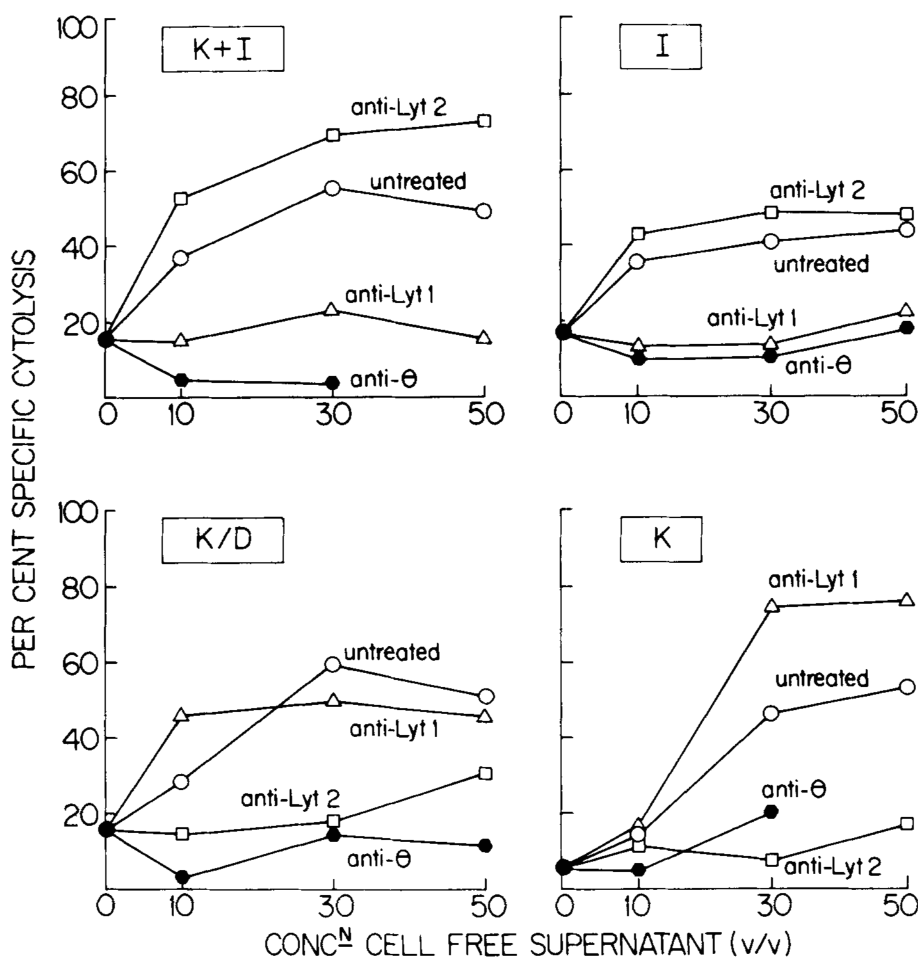

Figure 3. B10.AQR spleen cells were treated with anti-Lyt 1.2 (△), anti-Lyt 2.2 (□), or with anti-Thy 1.2 (●——●) serum and C. 10^7 Residual viable cells were then cultured with 5×10^6 T cell-depleted B10.A(5R)$_{Mit}$ (*upper left*), B10.T(6R)$_{Mit}$ (*upper right*), B10.A(2R)$_{Mit}$ (*lower left*), or B10.A$_{Mit}$ spleen cells (*lower right*). All stimulator cells were treated with anti-Thy 1.2 serum and C to eliminate the possibility that stimulator cells might contribute to the production of amplifying factor(s). In control cultures, 10^7 B10.AQR spleen cells were untreated (○——○) or treated with C alone. These two groups were indistinguishable and thus only the untreated group is shown. After 36 hr, cellfree supernatants were harvested from all cultures and the amplifying activity assayed as described in the text. The data shown were obtained by using several concentrations (range 10 to 50% v/v) of supernatant. Cultures were assayed for cytotoxic activity by using P815 target cells in a 4-hr ^{51}Cr release assay at an effector cell to target cell ratio of 50:1.

stimulator cells were exposed to UV-irradiation (Tables IV, V). The induction of cytotoxic responses to UV-irradiated stimulator cells was seen, however, after the addition of MLC supernatants containing augmenting factors (Table IV). This latter observation, previously made in circumstances involving stimulation across the entire MHC (10), has thus been shown to hold for both K/D and I region-encoded specificities. It is clear, therefore, that both K/D and I region-encoded specificities are able to induce CTL formation and to "trigger" the formation of soluble mediators.

Perhaps the most interesting result of the present study is the observation (Fig. 3) that the cellular requirements for mediator production differ with the alloantigenic stimulus involved. In studies involving stimulation across the entire MHC, we have previously shown that the production of amplifying factor(s) in primary MLC was dependent upon the presence of Lyt 1+ cells, and did not require Lyt 2+ cells (10). A similar finding in alloantigen-primed cell populations has been made by Wagner and Rollinghoff (8). The studies described in Figure 3 show that mediator production to I region (or to K + I) stimulation likewise showed a requirement for Lyt 1 +2− cells. In surprising contrast, however, mediator production to K/D region stimulation was not affected by removal of Lyt 1+ cells, but was almost totally ablated when Lyt 2+ cells were removed.

These findings show a striking parallel to recent studies by Swain *et al.* on the Ly phenotype of "allohelper" cells in the antibody response to sheep erythrocytes (25, 26). Helper cells generated in response to incompatibilities across the whole MHC, or to I region differences, were shown to be Lyt 1+2−, whereas helper cells generated across a K/D difference were found to be Lyt 1+2+.

In the context of cytotoxic responses to K/D stimuli, there have been several recent studies examining the Ly phenotype of the T cell populations involved. Melief *et al.* (27) found that in C57BL/6 mice stimulated with cells from an H-2K^{ba} mutant, maximal generation of Ly 1−2+ cytotoxic T cells required the presence of Ly 1+2− "helper" cells. In contrast, Bach and Alter (13), using H-2 recombinant mice, found that Lyt 1+2+3+ cells were necessary for the production of cytotoxic responses to K/D stimuli. However, the cytotoxic effector T cells in this study were also of a Lyt 1+2+3+ phenotype (28), making analysis of the helper cell population difficult. Observations similar to those of Bach were reported by Wettstein *et al.* (11, 12). The latter investigators used H-2 K/D mutant mice to demonstrate that proliferative responses, in an MLC involving I region compatibility but K/D differences, principally involved Lyt 1+2+ cells and that the cytotoxic cells arising in such cultures were also of this phenotype (11, 12). In these cases, however, it is not clear what role helper T cells (or their soluble mediators) played in the differentiation of cytotoxic T cells.

The report of Melief *et al.* (27) appears contrary to the observations presented here, for we have not been able to demonstrate a role for Lyt 1+ cells in mediator production to K/D region stimuli, even though, under the same experimental conditions, we could readily implicate such cells in responses to I region products. It seems possible that strain differences in the quantitative display of Ly alloantigens could account for our failure to detect Lyt 1 alloantigen expression on helper cells

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responding to K/D stimuli or, alternatively, that the product of Lyt 2+ cells is necessary for activation of Lyt 1+2+ cells. In any case, one theme persists through most studies so far carried out on "helper" cell responses to K/D stimuli: such cells bear Lyt 2, and thus belong to a T cell subpopulation distinct from "classical" T helper cells. It will now be of considerable interest to define the phenotype of helper T cells in the cytotoxic response to the minor histocompatibility antigens and to H-Y and other antigens that are coded for outside the major histocompatibility complex.

Although, to date, we have only been able to define a requirement for cells of a particular Ly phenotype in mediator production, the findings are concordant with the hypothesis that amplifying mediator(s) are produced by Lyt 1+ "helper" cells during MLC involving I region (or K/D + I region) incompatibility, but by Lyt 2+ cells when K/D region stimulation occurs. It is clear that both K/D and I region products can stimulate mediator production and CTL formation and that the absence of I region products on stimulator cells does not preclude the production of soluble mediators. The findings reported here imply that soluble mediators play a role in the differentiation of cytotoxic T cells regardless of the nature of the alloantigenic stimulus, and are thus fully compatible with a two signal model of cytotoxic T cell differentiation.

The nature of the amplifying factors present in MLC supernatants have to date been defined in phenomenologic, rather than biochemical, terms. It remains of particular interest to ask whether the amplifying factor(s) whose production is dependent upon the presence of Ly 1+ T cells have the same physiologic and chemical properties as those dependent upon Ly 2+ T cells. It will be of further interest to ask whether the amplifying factors produced under varying circumstances act only on cells destined to become cytotoxic, or whether they are also able to modulate the expression of B cell responses. We are currently investigating these questions.

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