Immunology 1980 **41** 653

Activation of secondary cytotoxic lymphocytes by cell-free factors from *I*-region-primed and *D*-region-primed lymphocytes

S. KANO, K. OSHIMI, M. SUMIYA & N. GONDA *Department of Medicine, Division of Clinical Immunology, Jichi Medical School, Japan*

Accepted for publication 2 June 1980

Summary. Cell co-operation in the generation of secondary cytotoxic responses was studied by selectively sensitizing lymphocytes in mixed lymphocyte culture (MLC) across *I* or *D* region difference and by combining the primed lymphocytes in the secondary MLC. Secondary cytotoxic responses were induced in *D*-region-primed lymphocytes by restimulation with the original priming *D*-region antigens, by co-culturing with the *I*-region-primed lymphocytes in the presence of the priming *I*-region antigens, or by cell-free supernatants obtained 24 h after the restimulation of *D*-region-primed lymphocytes and *I*-region-primed lymphocytes. The active MLC supernatants produced by both *I*-region-primed and *D*-region-primed cells also induced accelerated proliferative responses in *D*-region-primed lymphocytes. Heat-treatment or ultraviolet irradiation of the stimulator cells eliminated the capacity of the cells to induce the production of CTL-helper factor in *I*-region-primed and *D*-region-primed lymphocytes. It was concluded that both *I*-region-primed and *D*-region-primed lymphocytes produce a cell-free factor which induces proliferation and secondary cytotoxicity in *D*-region-primed lymphocytes. The possible participation of *D*-region reactive helper T cells and *D*-region reactive cytotoxic T cells in the cytotoxic responses to *D*-region antigens in the absence of *I*-region difference is discussed.

Abbreviations: ATS, anti-mouse thymus antiserum; CTL, cytotoxic T lymphocytes; MLC, mixed lymphocyte culture.

Correspondence: Dr S. Kano, Department of Medicine, Division of Clinical Immunology, Jichi Medical School, Minamikawachi-machi, Tochigi 329-04, Japan.

0019-2805/80/1100-0653$02.00

INTRODUCTION

In the mixed lymphocyte culture (MLC), cytotoxic T lymphocytes (CTL) are generated mainly against the cell surface determinants encoded by *K* and *D* regions of the *H-2* complex (Alter, Schendel, Bach, Klein & Stimpling, 1973). Although the *K* or *D* region difference alone can induce strong skin graft rejection (Klein, 1975) and can generate CTL in MLC under certain experimental conditions (Nabholz, Vives, Young, Miggiano, Rijnbeck & Shreffler, 1974), additional *I* region disparity is necessary for an optimal cytotoxic response (Schendel, Alter & Bach, 1973). In MLC across *K/D* and *I* region differences, the generation of CTL requires the co-operation of two different subsets of T cells: Lyt-23$^+$ CTL precursors responding to the *K/D* region difference and Lyt-1$^+$ proliferating helper T cells reacting to the *I* region difference (Cantor & Boyse, 1975). The helper T cells were replaceable by soluble factors in MLC supernatants (Plate, 1976).

It is not clear whether cell co-operation is also required in cytotoxic responses to *K/D* region antigens in the absence of *I* region difference, to mutant H-2K and H-2D antigens, or to hapten-modified self antigens. This problem was approached by selectively

sensitizing lymphocytes *in vitro* with either *D*-region difference or *I*-region difference alone, and by determining whether the development of secondary anti-*D*-region cytotoxicity in *D*-region-primed lymphocytes was augmented by the MLC supernatant from *D*-region-primed lymphocytes as well as from *I*-region-primed lymphocytes.

It was demonstrated that the CTL-helper activity was detectable in MLC supernatant from *D*-region-primed lymphocytes as well as in MLC supernatant from *I*-region-primed lymphocytes. Production of active MLC supernatant by *D*-region-primed lymphocytes may provide an experimental approach for elucidating the role of cell cooperation in cytotoxic responses to *K*/*D* region antigens without *I* region differences.

MATERIALS AND METHODS

Mice

Eight- to sixteen-week-old mice of the following strains were used in these experiments: A.TL, A.TH, B10.A, B10.A(2R), C57BL/6, DBA/2 and (B10.A × A.TL)F_1 hybrid. C57BL/6 and DBA/2 mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Sizuoka, Japan). The other mice were produced in our colony at Jichi Medical School, Tochigi, Japan. Breeding pairs of B10 congenic mice were gifts from Dr K. Moriwaki, National Institute of Genetics, Mishima, Japan. Breeding pairs of A.TL and A.TH mice were sent from Dr B. R. Bloom, Albert Einstein College of Medicine, N.Y., U.S.A.

Target cells

EL-4 (H-2^b) lymphoma cells, P 815 (H-2^d) mastocytoma cells and L-929 (H-2^k) fibroblasts were maintained *in vitro* in Joklik-modified Eagle's MEM supplemented with 5% foetal calf serum (Flow, Rockville, Md) and used as target cells in the ^{51}Cr-release assay.

Antiserum

The globulin fraction of rabbit anti-mouse thymus antiserum (ATS), absorbed with nude mouse spleen cells, was a gift from Dr M. Tanabe, Department of Microbiology, Jichi Medical School. Under the experimental conditions described in the Tables 3 and 5, ATS and complement-treatment killed less than 5% of nude mouse spleen cells and about 20% of normal mouse spleen cells.

Mixed lymphocyte culture

RPMI 1640 (Gibco, H-18), supplemented with Eagle's MEM essential and non-essential amino acids (Flow, Rockville, Md), penicillin (100 units/ml), streptomycin (100 μg/ml), 5×10^{-5} M 2-mercaptoethanol and 5% foetal calf serum (Flow, Rockville, MD), was used for lymphocyte cultures.

Primary MLC were established by mixing 100×10^6 responding spleen cells with an equal number of mitomycin C-treated stimulating spleen cells in 60 ml of the culture medium in 250 ml tissue culture flasks (Falcon, Div. Becton-Dickinson, Oxnard, CA. 3024). The flasks were incubated upright at 37° for 14 days in humidified air containing 5% CO_2.

Secondary MLC were made by mixing 3×10^6 viable cells, which had been harvested after 14 days of primary MLC, with an equal number of fresh mitomycin C-treated stimulating spleen cells in 2 ml of the culture medium in 16 mm flat-bottom wells (Linbro Scientific, Inc., Hamden, CT, FB-16-24-TC).

Production of MLC supernatants

Three million viable cells, which had been harvested after 14 days from the primary MLC and washed twice, were restimulated with an equal number of priming allogeneic spleen cells in 2 ml of the culture medium in 16 mm flat-bottommed wells for 24 h. At the end of the incubation period, culture supernatants were transferred to centrifuge tubes, centrifuged for 15 min at 500 *g*, and filtered through 0·45 μm millipore filters. The supernatants were tested immediately or stored at −20°.

Test for activity of MLC supernatants

(B10.A × A.TL)F_1 lymphocytes primed with B10.A(2R) (*D* region difference) were harvested after 14 days of primary MLC, washed twice and resuspended in a fresh culture medium. Aliquots of 4×10^5 viable cells in 0·1 ml of the culture medium were distributed in round-bottommed microculture wells (Linbro Scientific, Hamden, CT, IS-MRC-96-TC). To each culture, a volume of 0·1 ml of MLC supernatant, fresh culture medium or medium containing concanavalin A (5 μg/ml) was added. After incubation for 2 days, 0·1 ml of supernatant was carefully removed from each well and 0·04 ml of ^{51}Cr-labelled target cell suspension were added for cytotoxic assay.

Cytotoxic assay

Cytotoxicity was determined using a ^{51}Cr-release assay. EL-4 (H-2^b), P 815 (H-2^d), or L-929 (H-2^k)

Table 1. Co-operation between *D*-region-primed cells and *I*-region -primed cells in the generation of secondary cytotoxic responses

	Responding (B10.A × A.TL)F_1 cells				
Stimulating cells	B10.A(2R)-primed (3)	A.TH-primed (3)	B10.A(2R)-primed (3) + A.TH-primed (1)	B10.A(2R)-primed (3) + Normal (1)	Normal (3) + A.TH-primed (1)
(B10.A × A.TL)F_1(3)	17·5 ± 0·7	2·5 ± 0·3	17·6 ± 0·8	17·1 ± 2·1	1·2 ± 1·7
B10.A(2R) (3)	40·5 ± 3·9**	1·2 ± 1·7	35·7 ± 3·2**	36·8 ± 3·2**	1·1 ± 0·7
A.TH (3)	18·5 ± 1·3	4·4 ± 2·9	24·5 ± 2·3*	18·4 ± 1·3	1·5 ± 1·2
B10.A(2R) (1·5) + A.TH (1.5)	45·3 ± 1·9**	8·9 ± 5·2	42·7 ± 3·1**	42·0 ± 3·0**	1·7 ± 1·3

(B10.A × A.TL)F_1 mouse spleen cells were sensitized *in vitro* to either B10.A(2R) or A.TH; B10.A(2R)-primed or A.TH-primed cells, or a mixture of both were restimulated for 2 days.

Values represent % specific ^{51}Cr release (mean ± SD of triplicate cultures) from EL-4(H-2^b) target cells; the effector to target ratio was 20:1. The numbers in parentheses represent the numbers of viable cells ($\times 10^{-6}$) cultured. *$P < 0{\cdot}01$; **$P < 0{\cdot}001$.

target cells ($2{\cdot}5 \times 10^6$) were labelled for 1 hr with 100 μCi [^{51}Cr]-Na_2CrO_4, washed four times and resuspended in 5 ml of the culture medium. Effector cells harvested from secondary MLC were washed twice and resuspended in the culture medium. Because significant numbers of mitomycin-C treated stimulating cells were still viable on the second day of the secondary MLC and precise enumeration of the viable responding cells was not possible, the number of effector cells given in Table 1 refers to the number of viable responding cells originally cultured in the secondary MLC. Cytotoxic assays were carried out in round-bottomed microculture plates. 0·1 ml of effector cell suspension and 0·04 ml of a suspension of ^{51}Cr-labelled target cells were mixed and incubated for 4 h. The radioactivity released into the supernatant was recovered using the Skatron Titertek Supernatant Collection System (Flow, Rockville, MD). Spontaneous release (SR) represents the ^{51}Cr released in the supernatant from 2×10^4 target cells incubated in medium alone, and maximum release (MR) is the ^{51}Cr released by 2×10^4 detergent-lysed target cells. Results are expressed as percentage specific ^{51}Cr release calculated as:

$$\frac{\text{(c.p.m. experimental release} - \text{c.p.m. SR)}}{\text{(c.p.m. MR} - \text{c.p.m. SR)}} \times 100$$

DNA synthesis

Aliquots of 2×10^5 viable B10.A(2R)-primed or normal (B10.A × A.TL)F_1 lymphocytes in 0·1 ml culture medium were distributed in round-bottommed microculture wells. To each culture, a volume of 0·1 ml of MLC supernatnant, fresh culture medium, or medium containing concanavalin A (5 μg/ml) was added. The cultures were incubated for 2–6 days at 37° in humidified air containing 5% CO_2. The cultures were pulsed with [^3H]-thymidine (0·2 μCi/well) for the last 6 h, and harvested using a multiple sample harvester.

Concanavalin-A induced conditioned medium

Two hundred million C57BL/6 spleen cells were cultured in 40 ml of the culture medium containing 2·5 μg/ml of concanavalin A for 2 days. The culture medium was then cleared of cell debris by centrifugation (2000 r.p.m. for 15 min) and millipore filtration (0·45 μm), and was stored at −20°.

RESULTS

Selective sensitization to *I* region or *D* region difference

(B10.A × A.TL)F_1 mouse spleen cells were either sensitized to B10.A(2R) (*D* region difference) or A.TH (*I* region difference), or cultured with (B10.A × A.TL)F_1 cells for 14 days. Proliferative responses of sensitized lymphocytes were determined on day 2 of restimulation with B10.A(2R), A.TH or (B10.A × A.TL)F_1 stimulating cells. A rapid proliferative response was observed when either *I*-region-primed cells or *D*-region-primed cells were restimulated with the corresponding priming strain, showing the successful achievement of selective sensitization in these experiments (Fig. 1).

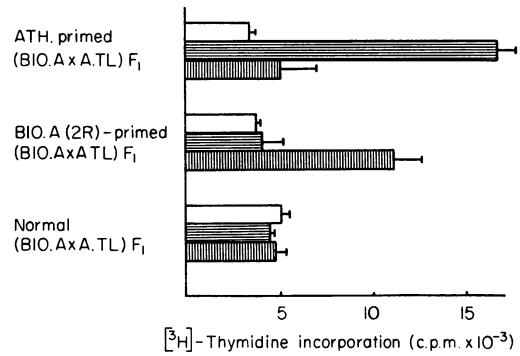

Figure 1. Selective sensitization to *D* or *I* region difference. (B10.A × A.TL)F_1 spleen cells which had been sensitized *in vitro* to A.TH (*I* region difference) or B10.A(2R) (*D* region difference), or normal (B10.A × A.TL)F_1 spleen cells were restimulated with mitomycin-C treated A.TH (horizontal hatching), B10.A(2R) (vertical hatching) or (B10.A × A.TL)F_1 (open columns) spleen cells for 2 days. [^{3}H]-Thymidine was added for the last 6 h. Values represent the mean ± SD of triplicate cultures.

Co-operation between *I*-region-primed and *D*-region-primed cells in the generation of the secondary cytotoxic responses

(B10.A × A.TL)F_1 mouse spleen cells primed with B10.A(2R) or A.TH, or a mixture of both were restimulated with B10.A(2R), A.TH or B10.A(2R) plus A.TH. Cytotoxic activity against EL-4 (H-2^b) target cells was assessed on day 2 of restimulation (Table 1). Restimulation of B10.A(2R)-primed (B10.A × A.TL)F_1 cells with B10.A$(2R)_m$ gave good secondary cytotoxic responses against EL-4 target cells, whereas restimulation with A.TH_m did not give a significant cytotoxic response. A.TH-primed (B10.A × A.TL)F_1 cells did not show any cytotoxic activity against EL-4 target cells. A mixture of B10.A(2R)-primed and A.TH-primed (B10.A × A.TL)F_1 cells showed significant cytotoxic response against EL-4 target cells when restimulated with A.TH_m in the absence of B10.A $(2R)_m$ stimulating cells. B10.A(2R)-primed (B10.A × A.TL)F_1 cells supplemented with normal fresh (B10.A × A.TL)F_1 spleen cells did not develop significantly increased cytotoxic activity when stimulated with A.TH_m. Nor did A.TH-primed (B10.A × A.TL)F_1 cells enhance the cytotoxic response of normal (B10.A × A.TL)F_1 spleen cells even when stimulated with B10.A$(2R)_m$ and A.TH_m. Similar results were obtained in five additional experiments.

It is therefore clear that: *D*-region-primed lymphocytes are necessary for secondary cytotoxic response; *D*-region-primed lymphocytes can be activated directly by the priming *D* region antigen or via the co-operative effect of *I*-region-primed lymphocytes which are restimulated with the priming *I* region antigens; normal fresh spleen cells stimulated with *I* region difference cannot replace the *I*-region-primed lymphocytes in exerting a co-operative influence at the early stage of secondary MLC.

Reactivation of the secondary cytotoxic lymphocytes is mediated by MLC supernatants from *I*-region-primed and *D*-region primed lymphocytes

Since *D*-region-primed lymphocytes can be activated by the co-operative influence of the *I*-region-primed lymphocytes, and the induction of secondary cytotoxic responses was shown to be mediated by supernatants from the MLC (Ryser, Cerottini & Brunner, 1978), it is suggested that the co-operative influence of the *I*-region-primed lymphocytes might be mediated by the active factors in MLC supernatants produced by the *I*-region-primed lymphocytes upon restimulation with the original sensitizing *I*-region antigens. In order to test this possibility, A.TH-primed, B10.A(2R)-primed or normal (B10.A × A.TL)F_1 spleen cells were stimulated with A.TH_m, B10.A$(2R)_m$ or (B10.A × A.TL)F_{1m} for 24 h, and MLC supernatant from each culture was tested to see whether it induces secondary cytotoxic response from B10.A(2R)-primed (B10.A × A.TL)F_1 lymphocytes against EL-4 target cells. Results are shown in Table 2.

MLC supernatant from A.TH-primed (B10.A × A.TL)F_1 cells restimulated with A.TH_m showed strong CTL-helper activity. Lesser but significant CTL-helper activity was found in MLC supernatant from B10.A(2R)-primed (B10.A × A.TL)F_1 cells restimulated with B10.A$(2R)_m$. MLC supernatant from A.TH-primed (B10.A × A.TL)F_1 cells stimulated with B10.A$(2R)_m$ or from B10.A(2R)-primed (B10.A × A.TL)F_1 cells stimulated with A.TH_m did not show CTL-helper activity. Supernatant from MLC in which normal (B10.A × A.TL)F_1 lymphocytes were stimulated with A.TH_m or B10.A$(2R)_m$ did not show any CTL-helper activity under the experimental conditions used in this study.

T cells required for the production of active MLC supernatants

A.TH-primed (B10.A × A.TL)F_1 lymphocytes were

Table 2. Activation of secondary cytotoxic lymphocytes by cell-free MLC supernatants from *I*-region-primed and *D*-region-primed lymphocytes

B10.A(2R)-primed (B10.A × A.TL)F_1 cells cultured with[a]		% Specific ^{51}Cr release[c] Exp. 1	Exp. 2	Exp. 3	Exp. 4
Culture medium		17·9 ± 2·7	6·3 ± 2·8	11·0 ± 1·5	10·5 ± 1·9
Concanavalin A		42·6 ± 4·6**[d]	42·5 ± 4·8**	48·5 ± 3·5**	57·2 ± 4·2**
MLC supernatant from[b]					
A.TH-primed (B10.A × A.TL)F_1	+(B10.A × A.TL)F_{1m}	15·0 ± 0·5	2·0 ± 0·6	11·6 ± 0·3	8·7 ± 1·0
	$A.TH_m$	28·5 ± 3·6*	28·3 ± 0·8**	34·8 ± 2·3**	19·0 ± 2·6*
	$B10.A(2R)_m$	17·4 ± 3·0	2·7 ± 2·9		
B10.A(2R)-primed (B10.AxA.TL)F_1	+(B10.AxA.TL)F_{1m}	10·5 ± 3·0	1·0 ± 1·4	9·4 ± 0·9	9·3 ± 0·6
	$A.TH_m$	14·8 ± 1·3	0·6 ± 1·9		
	$B10.A(2R)_m$	22·1 ± 1·2*	9·1 ± 1·1*	17·9 ± 1·7*	21·6 + 1·4**
Normal (B10.AxA.TL)F_1	+(B10.AxA.TL)F_{1m}	13·3 ± 1·2	3·7 ± 1·1		
	$A.TH_m$	15·9 ± 2·2	6·4 ± 2·2		
	$B10.A(2R)_m$	17·6 ± 1·3	0·4 ± 3·3		

[a](B10.A × A.TL)F_1 mouse spleen cells were sensitized *in vitro* with B10.A(2R). Viable lymphocytes which had been harvested from primary MLC (4×10^5/0·1 ml) were cultured with 0·1 ml of culture medium, medium containing concanavalin A (5 μg/ml), or MLC supernatants in round-bottommed microculture plate for 2 days. ^{51}Cr-labelled EL-4 (H-2^b) target cells (2×10^4) were then added for cytotoxic assay.

[b]MLC supernatants were obtained 24 h after restimulation of A.TH-primed, B10.A(2R)-primed, or normal (B10.A × A.TL)F_1 lymphocytes with stimulator cells described above.

[c]Results were expressed as mean ± SD of triplicate cultures.

[d]*$P < 0.01$, **$P < 0.001$.

treated with rabbit anti-mouse thymocyte globulin or normal rabbit serum plus complement before restimulation with A.TH spleen cells. MLC supernatants obtained after 24 h were then added to B10.A(2R)-primed (B10.A × A.TL)F_1 lymphocyte cultures. Production of active supernatants was completely abrogated by ATS and complement treatment, suggesting that active CTL-helper factor was produced by T lymphocytes (Table 3).

The effects of ultraviolet-light or heat treatment of stimulating cells on the production of active supernatants

Heat treatment or ultraviolet irradiation of the stimulator cells was shown to eliminate the capacity of the cells to stimulate a proliferative or cytotoxic response in MLC, while the capacity of the cells to serve as the targets of the cytotoxic lymphocytes remained intact (Lindahl-Kiessling & Safwenberg, 1971). In order to

Table 3. T cells required for the production of MLC supernatant

B10.A(2R)-primed (B10.A × A.TL)F_1 cells cultured with[a]		% Specific ^{51}Cr release[c] Exp. 1	Exp. 2
Culture medium		11·0 ± 1·5	13·3 ± 1·7
Concanavalin A		48·5 ± 3·5**[e]	66·4 ± 2·6**
MLC supernatant from[b]			
Untreated A.TH-primed (B10.A × A.TL)F_1	+(B10.A × A.TL)F_{1m}	11·6 ± 0·3	16·7 ± 1·1
Untreated A.TH-primed (B10.A × A.TL)F_1	$+A.TH_m$	34·8 ± 2·3**	34·1 ± 0·2**
(ATS+C)-treated A.TH-primed (B10.A × A.TL)F_1[d]	$+A.TH_m$	8·1 ± 1·2	14·8 ± 1·3
(NRS+C)-treated A.TH-primed (B10.A × A.TL)F_1	$+A.TH_m$	34·5 ± 2·6**	39·6 ± 2·1**

[a, b, c] Same as in Table 2.

[d] A.TH-primed (B10.A × A.TL)F_1 lymphocytes were treated with rabbit anti-mouse thymocyte serum (ATS) or normal rabbit serum (NRS) (final dilution 1:180) plus guinea-pig complement (final dilution (1:12) at 37° for 30 min, washed twice and then restimulated for 24 h.

[e] **$P < 0.001$.

Table 4. Effects of ultraviolet-light or heat treatment of stimulating cells on the production of active MLC supernatant

B10.A(2R)-primed (B10.A × A.TL)F_1 cells cultured with[a]		% Specific ^{51}Cr release[c]
Culture medium		11·0 ± 1·5
Concanavalin A		48·5 ± 3·5**[g]
MLC supernatant from[b]		
A.TH-primed (B10.A × A.TL)F_1	+ (B10.A × A.TL)F_{1m}	11·6 ± 0·3
	$A.TH_m$	34·8 ± 2·3**
	$A.TH_{heat}$[e]	12·5 ± 0·7
	$A.TH_{UV}$[f]	10·9 ± 0·9
B10.A(2R)-primed (B10.A × A.TL)F_1	+ (B10.A × A.TL)F_{1m}	9·4 ± 0·9
	$B10.A(2R)_m$	17·9 ± 1·7*
	$B10.A(2R)_{heat}$	11·9 ± 0·7
	$B10.A(2R)_{UV}$	8·2 ± 0·9

[a, b, c] Same as in Table 2.
[d] Mitomycin C (25 μg/ml) at 37° for 20 min.
[e] 45° for 30 min.
[f] 15 W UV lamp at 20 cm distance for 5 min.
[g] $*P < 0.01$, $**P < 0.001$.

test the effect of ultraviolet-light or heat treatment of stimulating cells on the production of CTL-helper factor, A.TH-primed and B10.A(2R)-primed (B10.A × A.TL)F_1 lymphocytes were restimulated with mitomycin-C treated, heat-treated (45°, 30 min), or ultraviolet-light treated (15W UV lamp at 20 cm distance, 5 min) original stimulator cells for 24 h, and MLC supernatants were then added to B10.A(2R)-primed (B10.A × A.TL)F_1 lymphocyte cultures for the determination of CTL-helper activity. Heat and ultraviolet treatment destroyed the capacity of the stimulator cells to induce the production of active supernatants in the *I*-region-primed and *D*-region-primed lymphocytes (Table 4).

Effect of MLC supernatant on T cells

The T-cell nature of B10.A(2R)-primed (B10.A × A.TL)F_1 responder cells is indicated by the data in Table 5. ATS plus complement treatment destroyed the capacity of B10.A(2R)-primed (B10.A × A.TL)F_1 responder cells to generate cytotoxic activity upon restimulation with active MLC supernatant. Trypsinization of responder lymphocytes to remove receptor-bound alloantigens did not affect the development of cytotoxic activity, suggesting that the activation of primed lymphocytes by the MLC supernatant is not dependent on the presence of alloantigens.

Table 5. Effect of MLC supernatant on alloantigen-primed T cells in the absence of alloantigens

	Pre-treatment of B10.A(2R)-primed (B10.A × A.TL)F_1 cells[a]			
Source of MLC supernatant	None	NRS + C	ATS + C	Trypsin
A.TH-primed (B10.A × A.TL)F_1 + (B10.A × A.TL)F_{1m}	16·7 ± 1·1[b]	12·1 ± 0·7	0·7 ± 0·2	14·1 ± 1·5
A.TH-primed (B10.A × A.TL)F_1 + $A.TH_m$	34·1 ± 0·2**[c]	27·1 ± 0·9**	2·4 ± 0·8	30·0 ± 2·3**

[a] B10.A(2R)-primed (B10.A × A.TL)F_1 lymphocytes (4×10^5) were treated with rabbit anti-mouse thymocyte serum (ATS) or normal rabbit serum (NRS) (final dilution 1:180) plus guinea-pig complement (final dilution 1:12) at 37° for 30 min, or treated with trypsin (0·25%) at 37° for 20 min, washed three times, and then cultured with MLC supernatant for 2 days. EL-4(H-2^b) target cells (2×10^4) were added to each culture for ^{51}Cr release assay.
[b] Values represent % specific ^{51}Cr release (mean ± SD of triplicate cultures).
[c] $**P < 0.001$.

Table 6. Specificity of secondary cytotoxicity induced by MLC supernatants

DBA/2-primed C57BL/6 lymphocytes cultured with[a]	% Specific ^{51}Cr release from target cells[c]		
	P815 (H-2^d)	EL-4 (H-2^b)	L-929 (H-2^k)
Culture medium	9·9 ± 2·5	0·6 ± 3·5	−1·2 ± 8·7
Concanavalin A	105·2 ± 4·5**[d]	−0·2 ± 3·7	2·8 ± 4·0
Concanavalin A-induced conditioned medium	92·5 ± 3·1**	−3·3 ± 0·8	3·0 ± 1·5
MLC supernatant from[b]			
A.TH-primed A.TL + $A.TL_m$	4·3 ± 1·9	−2·1 ± 1·9	−7·9 ± 6·2
A.TH-primed A.TL + $A.TH_m$	68·2 ± 10·0**	−0·5 ± 0·6	−7·7 ± 3·8
B10.A(2R)-primed B10.A + $B10.A_m$	7·2 ± 3·2	−4·4 ± 1·9	−18·0 ± 0·2
B10.A(2R)-primed B10.A + $B10.A(2R)_m$	47·5 ± 2·5**	−1·9 ± 3·5	−3·8 ± 3·4

[a] C57BL/6 mouse spleen cells were sensitized *in vitro* with DBA/2. Viable cells which had been harvested from primary MLC (4×10^5/0·1 ml) were cultured with 0·1 ml of culture medium, medium containing concanavalin A (5 μg/ml), concanavalin-A-induced conditioned medium, or MLC supernatants for 2 days. ^{51}Cr-labelled P 815 (H-2^d), EL-4 (H-2^b) or L-929 (H-2^k) target cells (2×10^4) were then added for cytotoxic assay.

[b] MLC supernatants were obtained 24 h after restimulation of the primed lymphocytes.

[c] Results were expressed as mean ± SD of triplicate cultures. Similar results were obtained in two additional experiments.

[d] $^{**}P < 0.001$.

Specificity of secondary cytotoxicity induced by MLC supernatants

Specificity of the secondary cytotoxic responses induced by MLC supernatant was investigated by using DBA/2 (H-2^d)-primed C57BL/6 (H-2^b) spleen cells as responding cells and testing on P 815 (H-2^d) mastocytoma cells, EL-4 (H-2^b) lymphoma cells and L-929 (H-2^k) fibroblasts as target cells. C57BL/6 spleen cells were sensitized *in vitro* with DBA/2 spleen cells. DBA/2-primed C57BL/6 lymphocytes were then cultured for 2 days in culture medium, culture medium containing concanavalin A, concanavalin-A induced conditioned medium, or MLC supernatant from B10.A(2R)-primed B10.A or A.TH-primed A.TL lymphocyte cultures. Results are shown in Table 6.

Secondary cytotoxicity induced by MLC supernatant from *I*- or *D*-region-primed lymphocytes was specific for the original priming alloantigen of the responding DBA/2-primed C57BL/6 lymphocytes. Little cytotoxicity was induced against third-party haplotype target cells or syngeneic target cells by MLC supernatant from *I*- or *D*-region-primed lymphocytes, and by concanavalin A or concanavalin-A-induced conditioned medium.

Blastogenic activity of MLC supernatant

MLC supernatants obtained by the restimulation of

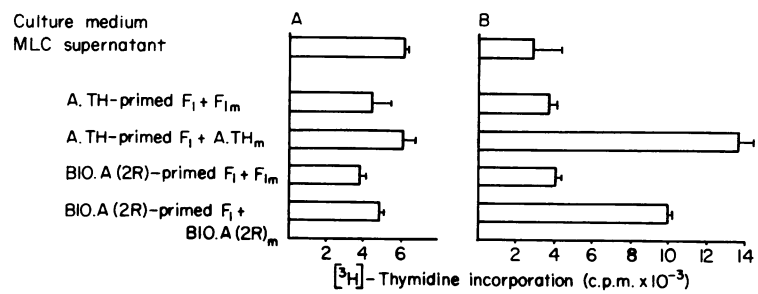

Figure 2. Blastogenic activity of MLC supernatant. Normal (B10.A × A.TL)F_1 (A) or B10.A(2R)-primed (B10.A × A.TL)F_1 (B) lymphocytes (2×10^5/0·1 ml) were cultured with 0·1 ml of MLC supernatant for 2 days. [^{3}H]-Thymidine was added for the last 6 h. Values represent the mean ± SD of triplicate cultures.

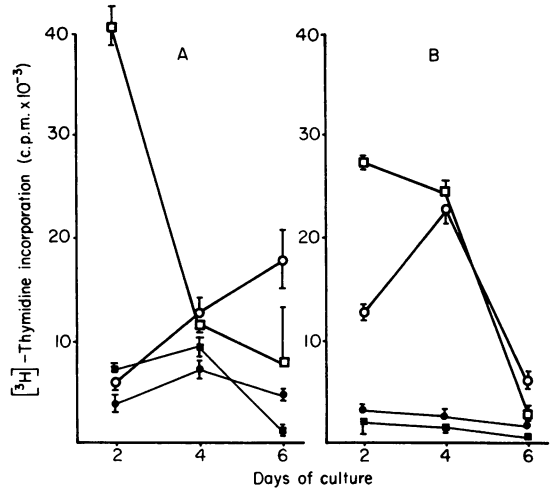

Figure 3. Kinetics of proliferative responses of normal (A) or B10.A(2R)-primed (B) (B10.A × A.TL)F_1 lymphocytes induced by MLC supernatant. (○) MLC supernatant from [A.TH-primed (B10.A × A.TL)F_1 + A.TH_m]; (●) MLC supernatant from [A.TH-primed (B10.A × A.TL)F_1 + (B10.A × A.TL)F_{1m}; (□) concanavalin A; (■) culture medium. Each point represents the mean of triplicate cultures (± SD).

A.TH- or B10.A(2R)-primed (B10.A × A.TL)F_1 lymphocytes with the original stimulator cells, were added to normal or B10.A(2R)-primed (B10.A × A.TL)F_1 lymphocytes. Cell proliferation was assayed 2 days later. As can be seen in Fig. 2, the MLC supernatants obtained from both A.TH-primed and B10.A(2R)-primed (B10.A × A.TL)F_1 lymphocytes upon restimulation with relevant antigens induced strong proliferative responses in B10.A(2R)-primed responder cells, but not in unprimed responder cells.

The kinetics of the proliferative responses induced by active MLC supernatant obtained from A.TH-primed (B10.A × A.TL)F_1 lymphocytes were determined using B10.A(2R)-primed and unprimed (B10.A × A.TL)F_1 cells. MLC supernatants induced a strong proliferative response in primed lymphocytes as early as day 2, with peak response on day 4 (Fig. 3). On the other hand, unprimed lymphocytes showed little proliferative response on day 2, slight but significant response on day 4 and strong proliferation on day 6. In contrast, proliferation induced by concanavalin A in unprimed cells was strongest on day 2 and sharply declined thereafter, but in B10.A(2R)-primed cells, concanavalin A induced sustained proliferation on days 2 to 4.

DISCUSSION

These studies were undertaken to determine the cellular co-operation and the role of different H-2 regions in the generation of secondary cytotoxic responses in the MLC. We approached these questions by selectively sensitizing lymphocytes in a primary MLC across *I* or *D* region difference alone and by combining the primed lymphocytes in the secondary MLC. The results reported here showed that secondary cytotoxic responses are induced in the *K*/*D*-region-primed lymphocytes by restimulation with the *K*/*D* different stimulating cells used in the primary MLC, by co-culturing with the *I*-region-primed lymphocytes in the presence of the priming *I*-region antigen, by stimulating with concanavalin A, or by the cell-free supernatant obtained from secondary MLC. While confirming the earlier observations by Alter, Grillot-Courvalin, Bach, Zier, Sondel & Bach (1976) that secondary CTL responses could be induced by restimulating MLC primed cells with stimulator cells sharing only the *I* region with the stimulating cells used in the primary MLC, and by Wagner & Rollinghoff (1978) and by Ryser *et al.* (1978) that cellular co-operation in the secondary MLC was mediated by a soluble product, we formally demonstrated the cellular co-operation between *K*/*D*-region-primed CTL and *I*-region-primed helper T cells in the secondary MLC.

The present study also revealed that active supernatant can be obtained through the restimulation of *D*-region-primed lymphocytes as well as *I*-region-primed lymphocytes. T cells are required for the production of CTL-helper factor, and ultraviolet-treated or heat-treated stimulating cells are not capable of stimulating active factor production.

While specific stimulation with the alloantigen used in the primary MLC is necessary for the production of the active MLC supernatant by the *D*-region-primed and the *I*-region-primed lymphocytes, once induced, the functional activity of the CTL-helper factor is not antigen specific or H-2 restricted, and the specificity of the secondary cytotoxicity is determined by the alloantigen used for primary sensitization of the primed CTL. The active MLC supernatants produced by both *I*-region-primed and *D*-region-primed cells induced secondary-type responses in the form of the proliferation of, and cytotoxicity in, *D*-region-primed T cells. The same MLC supernatants, however, gave little stimulation to unprimed spleen cells.

Whether the secondary cytotoxic response induced by the MLC supernatant is accounted for solely by the

proliferation of the small number of specific cytotoxic blast cells which are remaining at 14 days of the primary MLC or involves the reactivation of the memory cells was not assessed in this study. While the mitogenic activity of the MLC supernatant is considered more important, the latter possibility could not be excluded since MacDonald, Sordat, Cerottini & Brunner (1975) demonstrated the functional activation of memory cells in the absence of DNA synthesis in the early phase of the secondary MLC, and Wagner *et al.* (1978) reported the induction of cytolytic activity by semipurified secondary cytotoxic T-cell inducing factor (SCIF) in non-lytic (day 15) secondary MLC cells.

Supernatants from secondary MLC (Ryser *et al.*, 1978, Wagner & Rollinghoff, 1978) or primary MLC (Uotila, Ride & Gordon, 1978) were reported to induce antigen-specific secondary cytotoxic responses in the absence of antigen stimulation. Proliferating helper T cells were implicated by Ryser *et al.* (1978) and by Uotila *et al.* (1978) as being mainly responsible for the production of active supernatants. Wagner & Rollinghoff (1978) demonstrated that Lyt-1$^+$ cells release a factor which in turn triggers alloantigen-primed Lyt-23$^+$ cells to proliferation and cytotoxic activity. The results of the present study using *K*/*D* region compatible and *I* region incompatible strain combination are compatible with the view that Ia-reactive proliferating helper T cells are a major source of active CTL-helper factor. However, the results of the experiments using a *D*-region incompatible strain combination suggest that the *D*-region-primed T cells can also release a factor which induces proliferation and cytotoxic activity in alloantigen-primed lymphocytes.

Recently, it was proposed that a factor or factors which are produced by T cells and modulate lymphocyte activation be collectively called interleukin and factors which had been described as thymocyte-stimulating factor (TSF), thymocyte mitogenic factor (TMF), T-cell growth factor (TCGF), killer cell helper factor (KHF) or secondary cytotoxic T-cell inducing factor (SCIF) be designated Interleukin 2 (Aarden, Brunner, Cerottini *et al.*, 1979). Biological properties of the active factor produced by *D*-region-primed T cells are similar to those of interleukin 2. Whether this factor is biochemically identical to interleukin 2 remains to be determined.

In the present study, the lymphocytes which were primed *in vitro* to *D*-region difference without *I*-region-encoded differences displayed the following functions: a proliferative response to the priming *D* region antigen, cytotoxic activity against *D* region antigen, production of active CTL-helper factor, activation by active MLC supernatant, and activation by concanavalin A. Whether these activities are carried out by the same cell or by different subsets of lymphocytes is not clear. Recently, it was reported that it was possible to maintain cytotoxic T lymphocytes in a long-term culture with the help of T-cell growth factor obtained by concanavalin A or phytohaemagglutinin stimulation of normal spleen cells (Morgan, Ruscetti & Gallo, 1976; Gillis & Smith, 1977; Strausser & Rosenberg, 1978). These long-term cultured T-cell lines were induced to proliferation and cytotoxic activity by the addition of a T-cell growth factor. However, fresh culture medium supplemented with mitogenic doses of concanavalin A failed to induce proliferation in these T cells (Gillis, Fern, Ou & Smith, 1978; Rosenberg, Spiess & Schwarz, 1978). Therefore, the possibility is raised that the *D*-region-primed cytotoxic T cells are not directly stimulated by concanavalin A, but are activated indirectly via active factor(s) released by concanavalin A-stimulated helper T cells. The experiments reported here suggest similar cell co-operation mediated via cell-free factor(s) between *D*-region reactive cytotoxic T cells and *D*-region-primed helper T cells.

Helper T cells induced and reactivated by *K* and/or *D* region differences are not unique or exceptional to this experimental system. *K* and/or *D* region differences provide positive allogeneic effects in anti-sheep erythrocyte antibody production (Panfili & Dutton, 1978) and the helper activity in this system is dependent on the presence of Lyt-12$^+$ cells (Swain & Panfili, 1979). Co-operation between Lyt-1$^+$ helper cells and Lyt-23$^+$ CTL precursors was reported in the *in vitro* generation of cytotoxicity against a mutant H-2K difference (Melief, van der Meulen, Christiaans & de Greeve, 1979). T–T co-operation or the participation of soluble helper factors has been reported in the generation of cell-mediated cytotoxicity against syngeneic tumour cells, where the *I* region difference is apparently not involved (Glaser, 1979; Fyfe & Finke, 1979).

Heat treatment or ultraviolet irradiation of the stimulator cells eliminates the capacity of the cells to stimulate a proliferative or cytotoxic response in MLC, while the capacity of the cells to serve as the targets of the cytotoxic T lymphocytes remains intact (Lindahl-Kiessling & Safwenberg, 1971; Lafferty, Misko & Corley, 1974). Soluble helper factors, obtained from either MLC or concanavalin-A-stimu-

lated spleen cell cultures, have been shown to restore the ability of heat-treated or ultraviolet-irradiated cells to stimulate cytotoxic responses (Fyfe & Finke, 1979). Our results show that heat treatment or ultraviolet irradiation eliminates the capacity of stimulator cells to stimulate the production of active MLC supernatants from *I*-region-primed as well as *D*-region-primed cells and, together with above mentioned reports, suggest the participation of cell cooperation between helper T and cytotoxic T cells, via soluble mediators, in cytotoxic responses to *K/D* region antigens with or without *I* region differences.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Drs K. Moriwaki and B. R. Bloom for their generous supply of breeding pairs of mice, and to Dr M. Tanabe for his gift of anti-mouse thymus antiserum.

This work was partly supported by grants from the Ministry of Education, Culture and Science, and from the Ministry of Health, Japan.

REFERENCES

AARDEN L.A., BRUNNER T.K., CEROTTINI J.-C., *et al.* (1979) Revised nomenclature for antigen-nonspecific T cell proliferation and helper factors. *J. Immunol.* **123,** 2928.

ALTER B.J., GRILLOT-COURVALIN C., BACH M.L., ZIER K.S., SONDEL P.M. & BACH F.H. (1976) Secondary cell-mediated lympholysis: importance of *H-2* LD and SD factors. *J. exp. Med.* **143,** 1005.

ALTER B.J., SCHENDEL D.J., BACH M.L., KLEIN J. & STIMPLING J.H. (1973) Cell-mediated lympholysis. Importance of serologically defined H-2 regions. *J. exp. Med.* **137,** 1303.

CANTOR H. & BOYSE E.A. (1975) Functional subclasses of T lymphocytes bearing different Ly antigens. II. Cooperation between subclasses of Ly^+ cells in the generation of killer activity. *J. exp. Med.* **141,** 1390.

FYFE D.A. & FINKE J.H. (1979) Soluble helper factor(s) participates in the generation of cell mediated cytotoxicity directed against syngeneic tumor cells. *J. Immunol.* **122,** 1156.

GILLIS S., FERN M.M., OU W. & SMITH K.A. (1978) T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* **120,** 2027.

GILLIS S. & SMITH K.A. (1977) Long term culture of tumour-specific cytotoxic T cells. *Nature (Lond.)* **268,** 154.

GLASER M. (1979) T–T cell synergy in the *in vitro* generation of secondary cell-mediated cytotoxicity against syngeneic SV-40 transformed cells. *J. Immunol.* **122,** 973.

KLEIN J. (1975) *Biology of the Mouse Histocompatibility-2 Complex,* p. 172. Springer-Verlag, Berlin.

LAFFERTY K.J., MISKO I.S. & CORLEY M.A. (1974) Allogeneic stimulation modulates the *in vitro* response of T cells to transplantation antigen. *Nature (Lond.)* **249,** 275.

LINDAHL-KIESSLING K. & SAFWENBERG J. (1971) Mechanism of stimulation in the mixed lymphocyte culture. In: *Proceedings of the VIth Leukocyte Culture Conference* (Ed. by M. R. Schwarz), p. 623. Academic Press, New York.

MacDONALD H.R., SORDAT B., CEROTTINI J.-C. & BRUNNER K.T. (1975) Generation of cytotoxic T lymphocytes *in vitro*. IV. Functional activation of memory cells in the absence of DNA synthesis. *J. exp. Med.* **142,** 622.

MELIEF C.J., VAN DER MEULEN M.Y., CHRISTIAANS B.J. & DE GREEVE P. (1979) Cooperation between subclasses of T lymphocytes in the *in vitro* generation of cytotoxicity against a mutant H-2K difference. An analysis with anti-Lyt antiserum. *Europ. J. Immunol.* **9,** 7.

MORGAN D.A., RUSCETTI F.W. & GALLO R.C. (1976) Selective *in vitro* growth of T lymphocytes from normal bone marrows. *Science,* **193,** 1007.

NABHOLZ M., VIVES J., YOUNG H.M., MIGGIANO M., RIJNBECK A. & SHREFFLER D.C. (1974) Cell-mediated cell lysis *in vitro*: genetic control of killer cell production and target specificities in the mouse. *Europ. J. Immunol.* **4,** 378.

PANFILI P.R. & DUTTON R.W. (1978) Alloantigen-induced T helper activity. I. Minimal genetic differences necessary to induce a positive allogeneic effect. *J. Immunol.* **120,** 1897.

PLATE J.M.D. (1976) Soluble factors substitute for T–T collaboration in generation of T-killer lymphocytes. *Nature (Lond.),* **120,** 329.

ROSENBERG S.A., SPIESS P.J. & SCHWARZ S. (1978) *In vitro* growth of murine T cells. I. Production of factors necessary for T cell growth. *J. Immunol.* **121,** 1946.

RYSER J.-E., CEROTTINI J.-C. & BRUNNER K.T. (1978) Generation of cytotoxic T lymphocytes *in vitro*. IX. Induction of secondary CTL response in primary long-term MLC by supernatants from secondary MLC. *J. Immunol.* **120,** 370.

SCHENDEL D.J., ALTER B.J. & BACH F.H. (1973) The involvement of LD- and SD-region differences in MLC and CML: a three cell experiments. *Transplant. Proc.* **5,** 1651.

STRAUSSER J.L. & ROSENBERG S.A. (1978) *In vitro* growth of cytotoxic human lymphocytes. I. Growth of cells sensitized *in vitro* to alloantigens. *J. Immunol.* **121,** 1491.

SWAIN S.L. & PANFILI P.R. (1979) Helper cells activated by allogeneic *H-2K* or *H-2D* differences have a Ly phenotype distinct from those response to *I* differences. *J. Immunol.* **122,** 383.

UOTILA M., RIDE H.N. & GORDON J. (1978) Blastogenic factor: its role in the mixed leukocyte culture reaction. *Europ. J. Immunol.* **8,** 133.

WAGNER H. & ROLLINGHOFF M. (1978) T–T cell interactions during *in vitro* cytotoxic allograft responses. I. Soluble products from activated Lyl^+ T cells trigger autonomously antigen-primed $Ly23^+$ T cells to cell proliferation and cytotoxic activity. *J. exp. Med.* **148,** 1523.