GENERATION OF CYTOTOXIC T LYMPHOCYTES IN VITRO

I. Response of Normal and Immune Mouse Spleen Cells in Mixed Leukocyte Cultures*

By JEAN-CHARLES CEROTTINI, HOWARD D. ENGERS,‡ H. ROBSON MACDONALD,‡ and K. THEODOR BRUNNER

(From the Department of Immunology Swiss Institute for Experimental Cancer Research, 1011 Lausanne, Switzerland)

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It is well established that the immune response to alloantigens is characterized by the formation of effector cells which specifically destroy target cells carrying the sensitizing alloantigens in the absence of antibody and/or complement (for references, see 1). These effector cells belong to the thymus-derived (T) lymphocyte series and are generally referred to as cytotoxic T lymphocytes (CTL).[1]

With the development of a precise and reproducible cytotoxic assay system (2), it has been possible to follow the appearance of CTL during the primary and secondary response of mice to normal or tumor allografts. In certain instances, such studies have shown an earlier appearance and a higher peak of cytotoxic activity in spleens of mice injected twice with allogeneic tumor cells as compared to that observed after primary immunization, suggesting the existence of an anamnestic response in T-cell-mediated immunity (3–4).

Recently, it has been found that mouse CTL can be generated in vitro using mixed leukocyte cultures (MLC). Following the work of Häyry and Defendi (5), several studies have established the usefulness of the MLC system in providing an in vitro experimental model to analyze the series of events resulting in the appearance of CTL (6–10).

The present study was undertaken to investigate the effect of in vivo immunization on CTL generation in MLC. Using improved tissue culture conditions, we found that cell-mediated cytotoxic responses were significantly higher when the responding cells were obtained from immune (MLC-Imm) rather than normal spleens. At the peak of the response, MLC-Imm populations were five times more cytotoxic than MLC populations. Physical and immunological characterization of the effector cells generated in MLC-Imm gave results simi-

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[1] *Abbreviations used in this paper:* CTL, cytotoxic T lymphocytes; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; LU, lytic units; 2-ME, 2-mercaptoethanol; MLC, mixed leukocyte culture; MLC-Imm, MLC containing immune spleen cells as responding cells; MTLA, mouse T-lymphocyte antigen.

lar to those obtained for CTL formed in MLC, suggesting that the increased cytotoxicity of MLC-Imm was in fact related to a higher frequency of effector cells.

Materials and Methods

Mice.—Adult mice of the inbred strains C57BL/6, DBA/2, and C_3H/He were supplied by the animal colony maintained at the Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland. The breeding pairs for this colony were originally obtained from the Jackson Laboratories, Bar Harbor, Maine. AKR, BALB/c, and congenitally athymic "nude" C57BL/6 nu/nu mice were purchased from G1. Bomholtgard, Ry, Denmark.

Antisera.—Antiserum to theta alloantigen of C_3H mice (anti-θ) was prepared in AKR mice as described previously (11). The cytotoxic titer of this antiserum (as determined by the final dilution of antiserum which lysed 50% of 20,000 ^{51}Cr-labeled C_3H thymus cells in the presence of agar-absorbed normal rabbit serum as the source of complement) was 1:2,000. Rabbit antiserum to mouse immunoglobulin (Ig) was obtained after repeated subcutaneous and intramuscular injections of 1 mg Ig in complete Freund's adjuvant. As tested by immunodiffusion using myeloma proteins of different classes, this antiserum contained antibodies reacting with all mouse Ig classes. Sheep antiserum to rabbit IgG was obtained after three subcutaneous injections of 1 mg Fc fragment prepared according to Porter (12). Conjugation of antibody IgG fractions with fluorescein isothiocyanate (FITC) was carried out as described previously (13). The conjugates were then fractionated on diethylaminoethyl cellulose using the method of Cebra and Goldstein (14). Fractions with a FITC:protein ratio of 3–5 were selected for the immunofluorescent staining of living cells (13). Rabbit antiserum to mouse T-lymphocyte antigen (MTLA) was the generous gift of Doctors C. Bron and D. Sauser, Department of Biochemistry, University of Lausanne. Data regarding the specificity of this antiserum have been published previously (15).

Target cells.—Tumor cell lines syngeneic to DBA/2 (P-815-X2 mastocytoma, L-1210 lymphoma), to Balb/c (MOPC-315 plasmocytoma), and to C57BL/6 strains (EL4 leukemia, GIL4 lymphoma) were maintained in ascitic form in mice of the relevant strain and also in vitro (16). Target cells were labeled with ^{51}Cr by incubating 1–3 $\times$ 10^6 cells with 100–200 μCi radioactive sodium chromate (Eidg. Institut für Reaktorforschung, Würenlingen, Switzerland) in a final vol of 0.5 ml Tris phosphate-buffered saline (16) supplemented with 5% heat-inactivated fetal bovine serum (FBS, Flow Laboratories, Inc., Rockville, Md.). After incubation for 30–45 min at 37°C, the cells were washed three times with 10 ml Dulbecco's modified Eagle's medium (DMEM) containing 5% FBS and 10 mM *N*-Hydroxyethylpiperazine *N*-2-Ethanesulfonic acid (Hepes), pH 7.2.

Mixed Leukocyte Cultures (MLC).—Spleens of the appropriate strains were used as the source of responding and stimulating cells. Responding spleen cells were obtained from normal mice or from mice immunized 2–4 mo earlier with a single intraperitoneal injection of 30 $\times$ 10^6 living allogeneic tumor cells. As shown previously (17), the immune spleen cell populations exhibited very low cytotoxic activity remaining after the in vivo immunization. Lymphoid cell suspensions, prepared as described previously (2) were cultured in DMEM supplemented with L-asparagine (36 mg/liter), L-glutamine (216 mg/liter), L-arginine HCl (116 mg/liter), 5 $\times$ 10^{-5} M 2-mercaptoethanol (2-ME), and 5% FBS. This enriched medium will be subsequently referred to as MLC medium. Usually, 25 $\times$ 10^6 responding cells were mixed with an equal number of stimulating cells in 20 ml MLC medium in 30-ml tissue culture flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). Immediately before culture, the stimulating cells received a dose of 1,000 rads (Cesium source, Robatel, Genas, France) delivered at a dose rate of 827 rads/min. The culture flasks were incubated upright at 37°C in a humidified atmosphere of 5% CO_2 in air for the desired periods of time. Comparable results were obtained when 5 $\times$ 10^6 responding cells were cultured with 5 $\times$ 10^6 irradiated stimulating cells in 4-ml MLC me-

dium in flat-bottomed glass tubes (15 × 75 mm). At the end of the incubation period, the cells were collected by centrifugation, washed once with DMEM supplemented with FBS and Hepes, resuspended to 5×10^6 viable cells/ml as determined by hemocytometer counts using the trypan blue exclusion test, and then tested in the cytotoxic assay system described below.

Cell-Mediated Cytotoxic Assay.—The method of Brunner et al. (2) was modified as follows. A standard number of ^{51}Cr-labeled target cells (10×10^3) was mixed with varying numbers of MLC cells in a total vol of 0.4 ml DMEM supplemented with 5% FBS and 10 mM Hepes in round-bottomed plastic tubes (12 × 55 mm). The tubes were gassed with 5% CO_2 in air, sealed with plastic caps and either incubated in a shaking water bath (80 strokes/min) or incubated stationary in a water bath (following an initial centrifugation at 200 *g* for 60 sec). In practice, both procedures of incubation gave equivalent results. After incubation for 3 h at 37°C, 0.6 ml of cold phosphate-buffered saline was added to each tube, and the tubes were centrifuged at 600 *g* for 5 min. Then 0.5 ml of the supernatant fluid was carefully removed for measurement of radioactivity in a well type scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill.). Percent specific ^{51}Cr release was calculated from the formula described previously (16), taking into account ^{51}Cr spontaneous release (as measured with target cells incubated alone or with normal lymphocytes) and maximal release (as obtained after three cycles of freezing and thawing, or after incubation with a sufficient excess of CTL, whichever value was greater). Spontaneous ^{51}Cr release for the target cells used was in the range of 5–15% after 3 h of incubation, whereas maximal release was in the range of 75–95% of the total amount of isotope incorporated. For each lymphocyte population tested a dose-response curve was established, and the number of lytic units (LU) was calculated as indicated previously (16). In these experiments, 1 LU is arbitrarily defined as the number of lymphocytes required to achieve 50% lysis of 10×10^3 ^{51}Cr-labeled target cells within 3 h.

Characterization of Effector Cells.—

Treatment with anti-θ and complement: 50×10^6 cells were incubated with anti-θ serum (final dilution, 1:20) and agar-absorbed normal rabbit serum (final dilution, 1:12) in a total vol of 1.2 ml DMEM containing 5% FBS for 45 min at 37°C. Control cell suspensions were incubated with normal AKR serum and complement.

Filtration through Ig-anti-Ig columns: The method of Schlossman and Hudson (18) was modified as follows. Mouse Ig (obtained by the precipitation of normal serum with 40% saturated ammonium sulphate) was reacted with Sephadex G-200 beads (Pharmacia, Uppsala, Sweden) which had been activated with cyanogen bromide. An 8-ml vol of conjugate was packed in a 10-ml disposable syringe barrel fitted with a disc of sintered plastic. Rabbit serum directed against mouse Ig was passed over the column such that all the available Ig antigenic determinants became saturated with antibody. After washing, 20×10^6 lymphoid cells in DMEM supplemented with 20% FBS, 10 mM Hepes and 5 mM EDTA, pH 7.2, were applied to the column and eluted with the same medium.

Separation by velocity sedimentation at 1 g: The details of this technique have been described previously (19). In these experiments, a glass sedimentation chamber of 11 cm diameter was employed (Glassapparatebau, Weil, Germany). Cells to be separated were harvested from MLC, washed once in DMEM, and resuspended in DMEM supplemented with 3% (vol/vol) calf serum. 40 million cells in 30 ml were then applied to be buffered step gradient consisting of 7–30% calf serum in DMEM and allowed to sediment for 4 h at 4°C. After discarding the cone volume, 15-ml fractions were collected, concentrated to 1 ml by centrifugation, and then tested for cytotoxicity at various lymphocyte-target cell ratios, as described in the text.

RESULTS

Effect of 2-Mercaptoethanol on CTL Generation in MLC.—In a preliminary set of experiments, the effect of 2-mercaptoethanol (2-ME) on the formation of CTL during unidirectional MLC reactions was investigated. C57BL/6 spleen

cells (5×10^6) were cultured with irradiated (1,000 rads) DBA/2 spleen cells (5×10^6) in MLC medium with or without 5×10^{-5} M 2-ME. The use of this concentration of 2-ME was based on the results of preliminary studies in which 2-ME concentrations between 1×10^{-5} M and 2×10^{-4} M had been tested. On day 5, the number of living cells in individual cultures was determined and their cytotoxic activity was assayed using ^{51}Cr-labeled P-815 (DBA/2) tumor cells as target cells. It was found that the addition of 2-ME to the culture medium had two effects. First, cell recovery was increased almost twofold, reaching 2.8×10^6 cells (56% of the cell input) as compared to 1.5×10^6 (30% of the cell input) in the absence of 2-ME. Second, the cytotoxic activity of the cell population cultured in the presence of 2-ME was very much increased on a cell-to-cell basis (Fig. 1).

In order to take into account these two parameters, a quantitative estimation of the relative number of CTL per culture was necessary. Previous studies have indicated that such an estimation was feasible under well defined conditions (1, 9). To this end, the number of lymphoid cells required to achieve 50% lysis of 10×10^3 target cells within 3 h was calculated. As shown in Fig. 1, this number, which is further referred to as 1 LU, was equivalent to 25×10^3, or 270×10^3, cells in the case of MLC populations cultured in the presence, or in the absence, respectively, of 2-ME. When the number of LU per culture was calculated, the effect of 2-ME on the formation of CTL was particularly evident since 2-ME supplemented cultures contained 110 LU, as compared to 5 LU in cul-

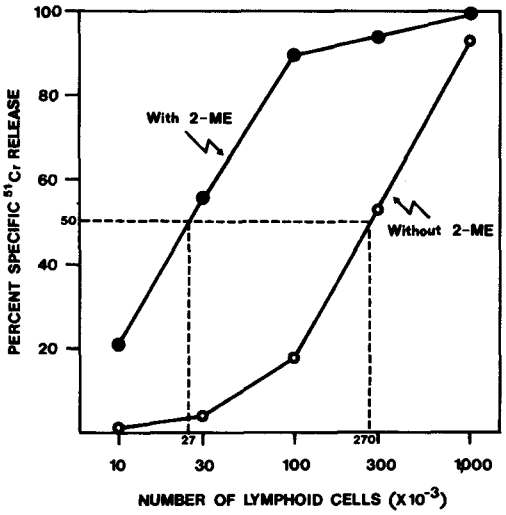

FIG. 1. Effect of 2-ME on the formation of CTL in MLC. Equal numbers of C57BL/6 spleen cells and irradiated DBA/2 spleen cells were cultured for 5 days in MLC medium with (●—●) or without (○—○) 5×10^{-5} M 2-ME. The cytotoxic activity of varying numbers of recovered cells was assayed on 10×10^3 ^{51}Cr-labeled P-815 (DBA/2) target cells (incubation time, 3 hr).

tures without 2-ME. Five additional experiments confirmed that inclusion of 2-ME in the MLC medium resulted in a 20–40-fold increase in the number of LU present in day 5 cultures. This enhanced cytotoxicity was not due to a direct effect on CTL activity since 2-ME, when added to the medium used in the cytotoxic assay, had no effect on the activity of CTL formed in MLC containing no 2-ME.

In order to test the specificity of the activity of CTL generated in vitro in the presence of 2-ME, C57BL/6 (*H-2*^{b}) spleen cells cultured with heavily irradiated DBA/2 (*H-2*^{d}) spleen cells for 5 days were tested for cytotoxicity on either allogeneic (*H-2*^{d}) or syngeneic (*H-2*^{b}) target cells. As shown in Table I, the two allogeneic target cells tested exhibited a similar susceptibility to lysis

TABLE I

*Cytotoxic Activity of MLC Populations**

Responding cells (*H-2*)	Stimulating cells (*H-2*)	Target cells (*H-2*)	Specific ^{51}Cr release				
			0.3:1‡	1:1	3:1	10:1	30:1
			%	%	%	%	%
C57BL/6(b)	DBA/2(d)	P-815(d)	12	32	71	98	95
		L-1210(d)	16	34	79	92	96
		EL4(b)	0	0	0	1	4
		GIL4(b)	0	1	2	2	1
BALB/c(d)	C57BL/6(b)	EL4(b)	8	21	44	72	92
		L-1210(d)	0	1	3	5	8
DBA/2(d)	C57BL/6(b)	EL4(b)	7	16	27	55	76
		P-815(d)	0	1	2	4	8

* Equal numbers of responding spleen cells and irradiated stimulating spleen cells were cultured (MLC). On day 5, the cytotoxic activity of the recovered cells was assayed on ^{51}Cr-labeled target cells (incubation time, 3 h).

‡ Lymphocyte: target cell ratio.

by CTL, whereas lysis of syngeneic target cells was very low, even at high lymphocyte: target cell ratios. These results were confirmed in further experiments, wherein the reverse MLC combinations were assayed for cytotoxicity on the same target cells (Table I, exp. 2). Analysis of dose-response curves indicated that 100 times fewer lymphocytes were required for lysis of the same number of allogeneic vs. syngeneic target cells.

Taken together, these results indicated that the greatly enhanced lytic activity observed in MLC populations was not due to the appearance of nonspecifically cytotoxic cells, or to the enhancement of CTL activity, but rather to an increase of the number of specific CTL generated under improved in vitro culture conditions. Therefore, 2-ME was included in the MLC medium used in all subsequent studies.

Kinetics of CTL Generation in MLC.—In order to study the time-course of CTL formation in MLC, C57BL/6 spleen cells were cultured with irradiated

DBA/2 spleen cells and tested for cytotoxicity on ^{51}Cr-labeled DBA/2 target cells at 24-h intervals. For each culture, both viable cell recovery and LU/10^6 cells were determined, and LU/culture were then calculated. The results are shown in Fig. 2. It can be seen that significant cytotoxicity of MLC populations was first demonstrable on day 2. From day 2 to day 4, the number of LU/culture increased about 100-fold and then remained at the peak level during the next 2

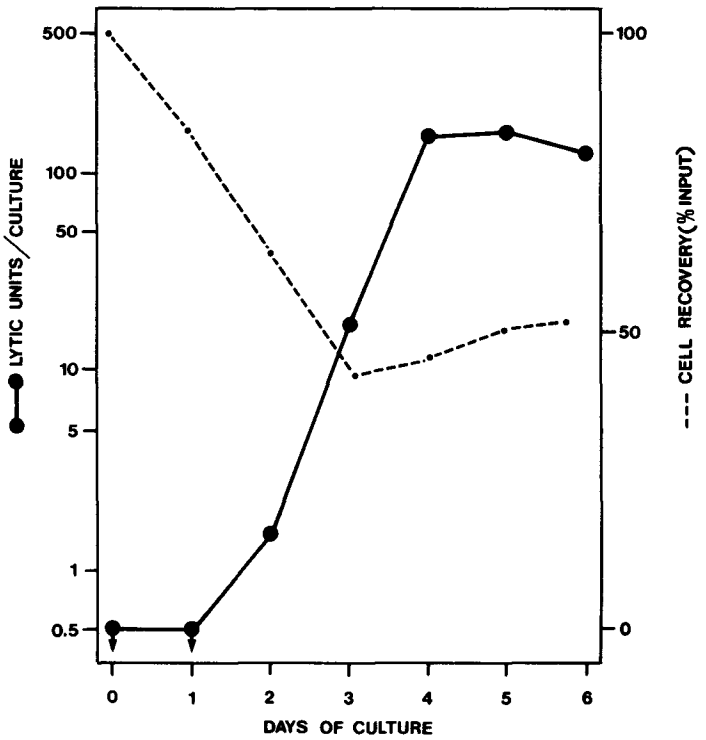

FIG. 2. Kinetics of CTL generation in MLC. At daily intervals, cultures of 5 × 10^6 C57BL/6 spleen cells and 5 × 10^6 irradiated DBA/2 spleen cells were assessed for viable cell recovery and assayed for cytotoxic activity on ^{51}Cr-labeled P-815 (DBA/2) target cells. LU/culture were calculated from the dose-response curve obtained with each population.

days. Viable cell recovery was around 50% at that time. Later on, the cytotoxic activity of cultures decreased progressively (data not shown), and by day 15 the number of LU/culture was less than one-tenth of the day 5 value, although viable cell recovery was still 10–20% of the cell input.

Effect of Immunization on CTL Generation in Vitro.—Previous studies (3–4) have demonstrated a secondary cell-mediated cytotoxic response in mice given two injections of allogeneic tumor cells 1–2 mo apart. This secondary response was characterized by earlier appearance, accelerated formation, and higher peak of CTL as compared to a primary response. We thus investigated whether or not a similar enhancement of cytotoxic activity could be demonstrated in vitro using

spleens from immunized mice as the source of responding cells in MLC (MLC-Imm).

C57BL/6 (*H-2*^{b}) mice were immunized with a single intraperitoneal injection of 30 × 10^6 P-815 (DBA/2) tumor cells. 2 mo later, i.e. at a time when the cytotoxic activity resulting from the primary response had almost completely disappeared, spleen cells were collected, cultured with irradiated DBA/2 (*H-2*^{d}) spleen cells for 4 days, and then assayed for cytotoxicity on *H-2*^{d} and *H-2*^{b} target cells. As a control, MLC containing spleen cells from normal C57BL/6 mice as responding cells were set up and assayed in parallel. The results indicated that in vitro sensitization of spleen cells from immunized mice gave rise to a highly cytotoxic cell population which was capable of killing 47% of 10 × 10^3 *H-2*^{d} target cells within 180 min at a lymphocyte:target cell ratio of 0.3:1. In comparison, approximately five times as many lymphocytes from primary MLC were needed to achieve the same degree of cytotoxicity.

The increased cytotoxicity of MLC-Imm populations was confirmed by the results of 15 different experiments carried out over a 12-mo period. When the number of LU/culture was calculated, a mean value ± SD of 135 ± 13 was found in day 4 MLC as compared to 670 ± 68 in day 4 MLC-Imm. This difference was even more striking at earlier times after the onset of the cultures. As shown in Table II, the difference in the number of LU/culture was 20-fold on day 2, and 13-fold on day 3.

Control experiments demonstrated that MLC-Imm populations exhibited a very low cytotoxic activity on syngeneic (*H-2*^{b}) target cells. The lack of nonspecific cytotoxicity was confirmed by the results of an "innocent bystander cell" experiment. Immune C57BL/6 spleen cells were cultured with irradiated

TABLE II

*Kinetics of the Generation of Cytotoxic Effector Cells in MLC and MLC-Imm Populations**

Day of culture	LU/culture‡	
	MLC	MLC-Imm
0	<0.5	2
1	<0.5	4
2	1.5	30
3	16	210
4	140	600
5	165	450
6	130	510

* 5 × 10^6 C57BL/6 responding (spleen) cells were cultured with 5 × 10^6 stimulating DBA/2 (irradiated spleen) cells. Responding cells were obtained either from normal mice (MLC) or from mice injected 2 mo previously with 30 × 10^6 P-815 (DBA/2) tumor cells intraperitoneally (MLC-Imm).

‡ Cytotoxic activity of the cultured populations was assayed on ^{51}Cr-labeled P-815 (DBA/2) target cells and LU were calculated from the dose-response curve obtained with individual cell populations.

DBA/2 spleen cells for 5 days, and assayed for cytotoxicity on either a mixture of ^{51}Cr-labeled C57BL/6 target cells and unlabeled DBA/2 target cells, or a mixture of ^{51}Cr-labeled DBA/2 target cells and unlabeled C57BL/6 target cells. Specific ^{51}Cr release was measured after incubation for 1 and 3 h. As indicated in Table III, lysis of the syngeneic (C57BL/6) bystander target cells remained low, even at supraoptimal lymphocyte:target cell ratios.

T-cell Nature of the Effector Cells Generated in MLC-Imm.—It is well established that the effector cells formed in MLC are T cells (8, 20–22). However, it is known that cell-mediated cytotoxicity in vitro can also be due to non-T-effector cells acting on target cells coated with IgG antibody (for reference, see 1). As mice immune to allogeneic tumor cells have been shown to produce IgG alloantibody upon secondary challenge (23–24), we thus investigated the possibility

TABLE III

*Specificity of the Cytotoxic Activity of MLC-Imm Populations**

Target cell mixture		Incubation time	Specific ^{51}Cr release			
Labeled cell	Unlabeled cell		0.3:1‡	1:1	3:1	10:1
		h	%	%	%	%
P-815 (*H-2^d*)	EL4 (*H-2^b*)	1	10	30	57	90
		3	57	96	99	100
EL4 (*H-2^b*)	P-815 (*H-2^d*)	1	0	0	1	1
		3	0	2	4	8

* Spleen cells from C57BL/6 (*H-2^b*) mice injected with 30×10^6 P-815 (*H-2^d*) tumor cells 5 mo earlier were cultured with irradiated DBA/2 (*H-2^d*) spleen cells (MLC-Imm). On day 5, the cytotoxic activity of MLC-Imm populations was assayed on a mixture of equal numbers of P-815 (*H-2^b*) and EL4 (*H-2^d*) target cells.

‡ Lymphocyte:target cell ratio.

that antibody-dependent cell-mediated cytotoxicity could be responsible, totally or partially, for the increased activity of MLC-Imm populations.

Lymphocyte populations obtained 4 days after incubation of immune C57BL/6 spleen cells with irradiated DBA/2 spleen cells were treated in two different ways before being assayed for cytotoxicity against DBA/2 target cells. Firstly, a cell aliquot was treated with anti-θ serum and complement for 45 min at 37°C. It was found that this treatment completely abolished the cytotoxic activity of the MLC-Imm population, whereas treatment with normal serum and complement had no effect. A second cell portion was filtered on a column of Sephadex G-200 beads coated with mouse Ig and rabbit antibody against mouse Ig (Ig-anti Ig). The unretained cell population was analyzed for its content of lymphocytes carrying either surface Ig or mouse thymus lymphocyte antigen (MTLA), and also tested for cytotoxicity. The same study was performed on MLC-Imm cells filtered on an Ig column treated with normal rabbit serum (Ig-NRS). The results are shown in Table IV. Before filtration, the MLC-Imm

TABLE IV

*Characterization of MLC-Imm Cells After Ig-Anti-Ig Column Filtration**

Cell characteristics	Type of column	
	Ig-NRS	Ig-anti-Ig
Surface Ig positive‡	11%	1%
MTLA positive§	83%	94%
LU/10^6 cells‖	111	125

* Spleen cells from immunized C57BL/6 mice were cultured with irradiated DBA/2 spleen cells (MLC-Imm). On day 4, cell aliquots of this MLC-Imm population were filtered through columns of Sephadex beads coated sequentially with mouse Ig and either normal rabbit serum (Ig-NRS) or rabbit serum to mouse Ig (Ig-anti Ig). Cell recovery was 53% on Ig-NRS column and 44% on Ig-Anti Ig column. Each eluate was assayed for cytotoxicity on ^{51}Cr-labeled P-815 (DBA/2) target cells.

‡ As determined by fluorescein-labeled rabbit IgG antimouse Ig.

§ As determined by rabbit serum to MTLA followed by fluorescein-labeled sheep IgG anti-rabbit Ig.

‖ LU were calculated from the dose-response curve obtained with each population.

population contained approximately 85% T cells and 10% Ig-positive cells (data not shown). This relationship was unchanged after filtration on the Ig-NRS column. However, after filtration on the Ig-anti Ig column, the percentage of Ig-positive cells was strongly reduced, and concomitantly, the percentage of T cells increased. Quantitative assessment of the lytic activity of both cell populations revealed a slight increase after filtration on the Ig-anti Ig column. Altogether, these results indicated that the cytotoxic activity of MLC-Imm cells was primarily mediated by T cells with little, if any, participation of non-T-effector cells.

Physical Characteristics of CTL in MLC-Imm.—Previous studies clearly showed that the majority, if not all, of CTL present in day 5 MLC populations were medium to large lymphocytes (22). In order to investigate the physical characteristics of CTL generated in MLC-Imm, spleen cells from immunized C57BL/6 mice were cultured with irradiated DBA/2 spleen cells for 5 days, collected, and fractionated by velocity sedimentation at 1 *g* (19). This technique allows the separation of cells according to their size. The MLC-Imm population was thus divided into nine fractions. For each fraction, the viable cell number was determined and the cytotoxicity quantitatively assessed on DBA/2 target cells using various lymphocyte:target ratios. From the dose-response curves obtained, the number of LU/10^6 cells was calculated. The results, shown in Table V, indicated an almost linear increase of LU/10^6 cells with sedimentation velocity (i.e., cell size). As compared to the unfractionated population, the number of LU/10^6 cells was 2.6 times higher in the most rapidly sedimenting fraction, and, reciprocally, 16 times lower in the slowest sedimenting fraction. By taking into account the number of cells present in each fraction, the number of LU/fraction was calculated and plotted as a percentage of the total number

TABLE V

*Velocity Sedimentation Distribution of CTL in MLC-Imm**

Cell fraction	Mean sedimentation velocity	LU/10^6 cells‡
	mm/h	
1	7.2	333
2	6.2	248
3	5.4	143
4	4.8	108
5	4.4	87
6	4.0	56
7	3.6	29
8	3.2	10
9	2.6	8
Unfractionated	—	125

* Spleen cells from immunized C57BL/6 mice were incubated with irradiated DBA/2 spleen cells (MLC-Imm). On day 5, the recovered cell population was separated into nine fractions by velocity sedimentation at 1 g. Each fraction was assayed for cytotoxicity on ^{51}Cr-labeled P-815 (DBA/2) target cells.

‡ LU were calculated from the dose-response curve obtained for each individual fraction.

of LU recovered (Fig. 3). The distribution of LU was clearly dissociated from the distribution of nucleated cells. Less than 10% of the total LU were present in the cell population with a sedimentation velocity below 3.8 mm/h, which contained 45% of the total cells recovered. Reciprocally, 40% of the total LU were present in the cell population with a sedimentation velocity greater than 5.8 mm/h, which contained about 10% of the total cells recovered. These results thus indicated that the majority of CTL in MLC-Imm populations were intermediate to large-sized lymphocytes.

DISCUSSION

We have shown that the generation of CTL in mouse MLC is considerably enhanced in the presence of 2-ME. By using a quantitative method for assessing the relative number of CTL (expressed as LU) present in MLC populations, it was found that addition of 2-ME to the culture medium resulted in a 20–40-fold increase of the number of LU formed at the peak of the response. Although cell recovery was significantly higher in the presence of 2-ME, the major effect of the reagent was related to an increased cytotoxic activity on a cell-to-cell basis. A direct influence of mercaptoethanol on the performance of effector cells was ruled out by the observation that the cytotoxic activity of CTL generated in MLC in the absence of 2-ME was independent of the presence of the reagent during the 3-h assay period. Thus, in accordance with the results of other studies (25–28), it was evident that 2-ME had a beneficial effect during the induction phase, but further experiments are needed to establish whether the increased cytotoxicity is related to (*a*) the triggering of a higher number of CTL precursor

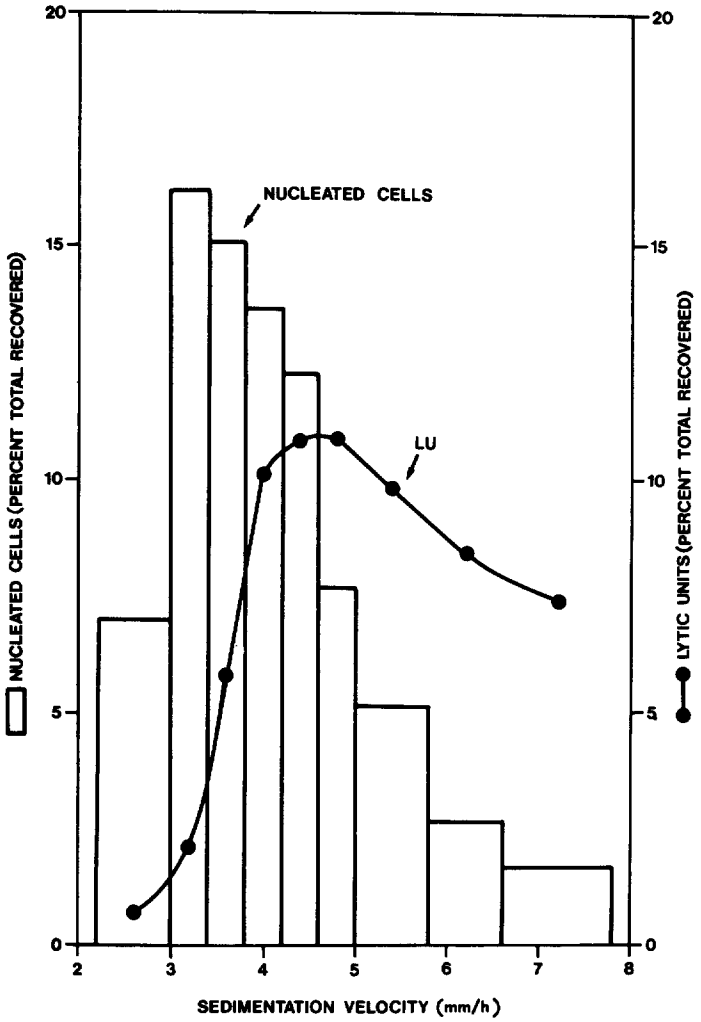

FIG. 3. Velocity sedimentation distribution of CTL in MLC-Imm. Spleen cells from C57BL/6 mice immunized against DBA/2 tumor cells were cultured with irradiated DBA/2 spleen cells for 5 days (MLC-Imm). Cells recovered from these cultures were originally separated into 14 fractions of equal volume according to their sedimentation velocity at 1 *g*. Certain fractions were combined to yield nine fractions as indicated in the figure. The distribution profile of nucleated cells and of lytic units is expressed in terms of the 14 original fractions.

cells, (*b*) an enhanced proliferation of responding cells, and/or (*c*) an effect of 2-ME on the differentiation of precursor cells into effector cells. Whatever the mechanism of action, it is clear that the increased cytotoxicity of MLC populations cultured in the presence of 2-ME is as specific as that of CTL formed either in vivo (4, 29) or in vitro in the absence of 2-ME (7–9).

As a result of this increased cytotoxicity, CTL were first detectable by day 2, that is, 24–48 earlier than reported in other studies using similar strain combinations and target cells (7–8). Also, at the peak of the response, the MLC population had a higher lytic activity than any in vivo-generated source of CTL, in agreement with the work of Wagner et al. (7). In particular, MLC cell populations were about 10 times more active than spleen cells from mice immunized with an optimal dose of DBA/2 tumor cells (30). Even peritoneal exudate cells collected from mice immunized by the intraperitoneal route, which have been found to contain a high frequency of CTL, were about two times less active than MLC populations (30).

An interesting feature of this work is the demonstration of an enhanced in vitro cytotoxic response of spleen cells from immunized mice (MLC-Imm). As compared to the data obtained with normal responding cells, the cytotoxic activity of MLC-Imm populations was detectable earlier and reached higher peak levels. Further experiments indicated that (*a*) cytotoxicity was highly specific, (*b*) the effector cells belonged to the T-lymphocyte series, and (*c*) their physical characteristics were very similar to those of CTL generated in MLC (22). It is thus likely that the difference in cytotoxic activity between MLC and MLC-Imm populations was directly related to the number of CTL generated under these conditions.

The enhanced in vitro cytotoxic response of spleen cells from immunized mice was in agreement with previous in vivo studies showing accelerated appearance and higher levels of cytotoxicity in spleens of mice injected twice with allogeneic tumor cells (3–4). It thus appears that memory can be demonstrated for this particular type of T-cell response. Several questions are raised by these observations. Firstly, it remains unsettled whether or not the secondary CTL response involves qualitative as well as quantitative differences, as is the case for humoral immunity. In this context, the existence of a qualitative difference between CTL precursor cells from either normal or immunized mice has been recently suggested by the finding that the latter cells are able to generate high numbers of effector cells in vitro in the presence of alloantigen membrane extracts, whereas normal spleen cells respond very poorly to the same antigenic stimulus.[2] Secondly, the origin of the CTL precursor cells present in spleens of immunized mice is unclear. The possibility exists that the pool of precursor cells has simply expanded as the result of primary immunization. Alternatively, the cells responding to a secondary stimulation may represent the progeny of effector cells generated during the primary response. Previous studies of the physical characterization of CTL (22, 31) have suggested the following differentiation pathway during the primary response in vivo: CTL precursor cells, which are dense, small lymphocytes, first differentiate into large, low-density effector cells. As the response proceeds, the latter cells further differentiate into dense, small

[2] Engers, H. D., K. Thomas, J.-C. Cerottini, and K. T. Brunner. 1974. Manuscript in preparation.

CTL, which slowly lose cytotoxic activity in the absence of antigenic stimulation. It is thus tempting to speculate that CTL may reach a stage of development where they are no longer cytotoxic, but will differentiate again into effector cells upon secondary stimulation. Since the detailed analysis of lymphocyte differentiation pathways is difficult in whole animal studies, support for this hypothesis must come from experiments in which primary and secondary CTL responses are induced in vitro. Studies in this direction are described in the following paper (32).

SUMMARY

Mouse cytotoxic T lymphocytes (CTL) were generated in mixed leukocyte cultures (MLC) using spleen cells as responding cells and irradiated allogeneic spleen cells as stimulating cells. Cytotoxicity was assessed by a quantitative ^{51}Cr assay system and the relative frequency of CTL in individual cell populations was estimated from dose-response curves. Inclusion of 2-mercaptoethanol in the MLC medium resulted in a 20–40-fold increase in the relative number of CTL generated at the peak of the response. Under these culture conditions, cell-mediated cytotoxic activity was detectable in MLC populations as early as 48 h after the onset of the cultures.

When spleen cells from mice immunized with allogeneic tumor cells 2–4 mo previously were cultured with irradiated spleen cells of the same alloantigenic specificity (MLC-Imm), it was found that the cell-mediated cytotoxic response was detectable earlier and reached higher levels than that observed in a primary MLC. At the peak of the response, MLC-Imm populations were observed to lyse up to 50% of the target cells within 3 h at a lymphocyte:target cell ratio of 0.3:1. Immunological and physical characterization of the effector cells generated in MLC-Imm indicated that they were medium to large-sized T lymphocytes. Altogether, these studies suggested the existence of an anamnestic cell-mediated cytotoxic response in MLC-Imm.

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