CHARACTERIZATION OF THE KILLER CELL GENERATED IN THE AUTOLOGOUS MIXED LEUKOCYTE REACTION*

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The proliferative response of human T cells cultured with autologous non-T cells is known as the autologous mixed lymphocyte reaction (AMLR)[1] (1–3). The stimuli for proliferation remain unclear, but probably include xenoantigens (4) Ia-like materials presented on dendritic, macrophage, B, or null cells (5–7). In the course of the AMLR, T cell subsets are expanded, which both helps autologous B cells to synthesize immunoglobulin (Ig) and demonstrates suppressor functions (8–10). Simultaneously, mitogenic factors, such as interleukin 2, are produced in the cultures (9, 11, 12).

There is disagreement about the generation of cytotoxic cells in the AMLR. Vande Stouwe et al. (13) observed that AMLR supernatants induced cells that killed allogeneic targets, but found no autologous cytotoxic T cells. Similarly, it has been reported that cytotoxic T cells do not develop in the murine syngeneic MLR (14). Miller and Kaplan (15) also were unable to demonstrate cytotoxic effector cells in an AMLR where the targets were autologous unstimulated mononuclear cells or purified B cells, but good killing of B lymphocytes when the targets were LPS-stimulated mononuclear cells. Subsequently, Tomonari (16) reported that cytotoxic T cells could be generated in the AMLR that lysed autologous targets, as well as various human lymphoid cell lines. The AMLR killers had some characteristics similar to natural killer (NK) cells. We too observed a lymphocyte-mediated cytotoxicity to a variety of targets in response to autologous stimulation, but the responsive cell lacked an Fc receptor for IgG and thus differed from conventional NK cells (17).

The present studies were performed to determine the conditions for the production of cytotoxic T cells in the autologous MLR, to define the surface phenotype of the responsible cells using monoclonal antibodies (MAb), and to distinguish them from other cytotoxic effector cells, such as NK (18–20) and cytolytic T lymphocytes (CTL) (21, 22).

Materials and Methods

Media. RPMI-10% fetal bovine serum (FBS) consisted of RPMI supplemented with 10% heat-inactivated FBS (Grand Island Biological Co., Grand Island, NY), 200 U penicillin/ml,

* Supported by grant AM-14916 from the U. S. Public Health Service, and grants from the Arthritis Foundation Center and the Kroc Foundation.

[1] *Abbreviations used in this paper:* AMLR, autologous mixed lymphocyte reaction; AP, autologous plasma; C′, complement; CTL, cytotoxic T cell; E^+, erythrocyte-rosetting T cells; E^-, non-erythrocyte-rosetting T cells; E/T ratio, effector/target cell ratio; FBS, fetal bovine serum; IL-2, interleukin 2; LAK, lymphokine-activated killer cells; MMC, mitomycin C; MAb, monoclonal antibody; NK, natural killer; PBL, peripheral blood lymphocytes; PBM, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; SRBC, sheep erythrocytes.

10 μg gentamycin/ml, and 300 μg L-glutamine/ml. RPMI-20% autologous plasma (AP) consisted of RPMI 1640 containing 20% heat-inactivated AP, 200 U penicillin/ml, 10 μg gentamycin/ml, 300 μg L-glutamine/ml, 25 mM Hepes/ml (Grand Island Biological Co.), and 0.05 mM 2-mercaptoethanol/ml (Mallinckrodt Inc., Science Products Div., St. Louis, MO).

Peripheral Blood Mononuclear Cell (PBM) Purification. PBM from healthy volunteers (22–54 yr old) of both sexes were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation (23). Cells aspirated from the interface were washed three times with RPMI-10% FBS. PBM (2×10^7/ml) were mixed with an equal volume of 10% neuraminidase-treated (24) sheep erythrocytes (SRBC) in SRBC-absorbed, heat-inactivated FBS. After centrifugation at 70 *g* for 5 min, the cell mixture was incubated at 37°C for 15 min and at 4°C overnight. Pellets were gently aspirated and layered over a second Ficoll-Hypaque gradient. The erythrocyte-rosetting T (E^+) cells were recovered in the pellet and the non-erythrocyte-rosetting (E^-) cells were collected from the interface. SRBC were lysed by hypotonic shock and both E^+ and E^- cell fractions were washed with RPMI-10% FBS three times. In some experiments in which fresh NK activity was assayed, monocytes were depleted of peripheral blood lymphocytes (PBL) by incubating PBM in FBS-coated plastic plates at 37°C for 1 h (25).

AMLR. AMLR cultures were performed in multi-well tissue culture plates (Linbro 76-033-05; Flow Laboratories, Inc., Rockville, MD) with 2.1×10^6/well E^+ cells and 2.1×10^6/well mitomycin C (MMC) (Sigma Chemical Co., St. Louis, MO) -treated E^- cells in 2.9 ml/well RPMI-20% AP. Non-T cells (E^-) were treated with 25 μg/ml of MMC for 60 min at 37°C followed by three washes in RPMI-10% FBS, and resuspended in RPMI-20% AP. Control cultures of E^+ cells alone and MMC-treated E^- cells alone at 2.1×10^6 cells/2.9 ml in RPMI-20% AP were included in all experiments. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Cell Viability and Proliferation of AMLR Cultures. Cells recovered from AMLR and control cultures were washed three times with RPMI-10% FBS and the number of viable cells was assessed by trypan blue dye exclusion. Proliferative responses were determined by incubating 2×10^5/0.2 ml of cells recovered from AMLR and control cultures and 1 μCi/well [^{3}H]-thymidine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, NY) for 18 h at 37°C in a humidified 5% CO_2 atmosphere. The plates were set up in triplicate in Linbro microtiter plates (76-001-05; Flow Laboratories, Inc.) and harvested on a multiple automatic sample harvester (Otto Hiller; Madison, WI). The amount of incorporated isotope was measured in a liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA), and results were expressed as the mean counts per minute of triplicate samples.

MAb. The characteristics of the individual MAb used in this study are presented in Table I. The OK reagents were from Ortho Diagnostic Systems, Westwood, MA; 9.3 and 9.6 from New England Nuclear, Boston, MA; the Leu-7 (HNK-1) from Becton, Dickinson & Co., Mountain View, CA. T101 and 4F2 were generously provided by I. Royston (University of California, San Diego) and A. Fauci (National Institutes of Health), respectively. As a control MAb, mouse IgG_{2a} myeloma protein RPC-5 (Bionetics Laboratory Products, Litton Bionetics, Kensington, MD) was used.

Complement (C′)-mediated Cell Lysis. 5×10^6 cells were incubated in 0.5 ml of culture medium containing the MAb at the final concentrations shown in Table I. Cells to be treated with C′ alone were incubated in culture medium without MAb. After a 1-h incubation at 22°C, carefully selected nontoxic rabbit C′ was added at a final dilution of 1:2, and the cells were incubated for 1 h at 37°C. The cells were then pelleted and the C′ was removed and replaced with 1 ml fresh C′ diluted 1:2. The cells were then incubated for another hour at 22°C, layered on modified Ficoll-Hypaque gradients and centrifuged to deplete the dead cells (20, 42). The cells at the interface of the gradients, consisting of 98% viable cells, were washed thrice with RPMI-10% FBS before cytotoxicity assay. The percentage of residual antigen-positive cells after C′ plus MAb treatment was usually <5%.

Preparation of Aggregated IgG. A 2% solution of rabbit IgG (Cohn fraction II; Miles Laboratories Inc., Elkhart, IN) was heat aggregated at 63°C for 30 min. Sodium sulfate (2.18 M) was added and the mixture was incubated at 4°C for 30 min, and then centrifuged at 3,000 *g* for 30 min. The sediment was resuspended in phosphate-buffered saline (PBS) and dialyzed

TABLE I
Characteristics of MAb

MAb	Specificity	Ig subclass	Antigen mol wt	Final concentration in C′-mediated lysis	Reference
			$\times 10^{-3}$		
9.3	Suppressor/killer T	IgG_{2a}	44	1:100	26–28
9.6	E^+	IgG_{2b}	50	1:100	27, 28
T101	Pan T	IgG_{2a}	65	1:1,000	29
OKT4	Helper/inducer T	IgG_{2b}	62	1:50	30–34
OKT6	Thymocyte	IgG_1	49	—*	30, 31, 34
OKT8	Suppressor/killer T	IgG_{2a}	76	1:50	30, 31, 34, 35, 38
OKT9	Early hematopoietic stem cell, activated T	IgG_1	94	—	30, 31, 34, 38
OKT10	Early hematopoietic stem cell, monocyte, null cell activated T, B	IgG_1	45	—	30, 31, 34, 38
OKM1	Monocyte, granulocyte, null cell	IgG_{2b}	173	1:50	36, 38
OKI_{a1}	Ia^+ B, monocyte, T	IgG_2	34	1:50	37, 38
$4F_2$	Monocyte, activated T	IgG_{2a}	120	1:100	39, 40
Leu-7	NK	IgM	NR‡	1:20	41

* Non-complement-fixing.
‡ Not reported.

extensively against PBS at 4°C. The solution was ultracentrifuged at 104,000 *g* for 90 min. The pellet was resuspended in PBS and centrifuged at 5,000 *g* for 30 min. The final supernatant was used as an aggregated IgG at concentrations ranging from 3 to 300 μg/ml.

Cell Surface Immunofluorescence. 1×10^6 cells were incubated on ice for 45 min with appropriate dilutions of the MAb (OKT4, OKT6, OKT8, OKT9, OKT10, OKIa1, and OKM1 diluted 1:100; 9.6 and 4F2 diluted 1:1,000; and Leu-7 diluted 1:20) in buffer consisting of RPMI, 5% gamma globulin-free horse serum (Grand Island Biological Co.), and 0.2% sodium azide. The cells were washed twice with the buffer and incubated on ice for 30 min with fluorescein-conjugated goat anti-mouse IgG or IgM (only for Leu-7) diluted 1:20 in buffer. Fc receptors for IgG were also detected by uptake of heat-aggregated rabbit IgG by incubating lymphocytes with 100 μg/ml of heat-aggregated IgG for 30 min at 4°C. After two washings, the cell surface-bound aggregate was visualized by incubating the cells with a fluorescein-conjugated goat anti-rabbit Fc antiserum for 30 min at 4°C (43). The fluorescein-conjugated reagents were purchased from N. L. Cappel Laboratories Inc., Cochranville, PA. After three washings with buffer, the cells were examined for cell surface immunofluorescence using a Zeiss fluorescent microscope with epi-illumination (Carl Zeiss, Inc., Thornwood, NY).

Target Cell Lines. The K562 erythroleukemic cell line (44) and two Burkitt lymphoma lines, Raji and Daudi, were grown in suspension culture in RPMI-10% FBS. The K562 cell line is sensitive to NK lysis (18) and both Raji and Daudi cell lines are resistant to NK lysis (45). Daudi is also resistant to CTL-mediated lysis (45, 46). Both K562 and Daudi lack HLA antigens (45).

Cytotoxicity Assay. Target cells were radiolabelled by incubating 5×10^6 cells with 0.05 ml $Na_2{}^{51}CrO_4$ (sp act 1.0 mCi/ml; New England Nuclear) for 2 h at 37°C with occasional shaking followed by at least three washings with the medium (18). Target cells (1×10^4 containing ~8–

20×10^3 cpm) in 0.1 ml medium were mixed with 200,000–25,000 viable effector cells in 0.1 ml media to give effector to target (E/T) ratios of 20:1 to 2.5:1, in wells of sterilized microtiter V-plates (Dynatech Laboratories, Alexandria, VA). The plates were centrifuged for 5 min at 60 *g* to facilitate the contact between the effector and target cells, and then incubated for 4 h at 37°C in humidified air plus 5% CO_2. After the incubation, the plates were centrifuged for 10 min at 150 *g*. An aliquot of the supernatant (100 μl) was removed from each well and counted in an automatic gamma scintillation counter. Spontaneous release of ^{51}Cr was assessed by incubating the target cells in media without the effector cells in the same procedure. In a typical experiment, spontaneous release was <5%. Maximum release was obtained by suspending the labeled target cells in 0.1 N HCl. All tests were performed in triplicate. Differences among percent release in triplicates were within 5%. The percentage of cytotoxic activity was calculated as follows: [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)] × 100. In the cytotoxicity assay described above, the effector cells in the NK test were fresh PBL, and the AMLR cytotoxic cells were E^+ cells obtained by overnight rosetting with SRBC at various times throughout the culture period for 3–12 d.

Blocking Experiments. The ability of either MAb or heat-aggregated IgG to block the cytotoxic activity of fresh NK- and AMLR-generated cells was tested in the following manner. MAb (10 μl) at 1.5 μg/ml (or 4F2 at 1:100 dilution) or aggregated IgG (3–300 μg/ml) was added to 1×10^5 (for AMLR killer activity) or 2×10^5 (for fresh NK activity) effector cells in the assay wells. This mixture was maintained at room temperature for 1 h, and chromium-labeled target cells (1×10^4) in a final volume of 200 μl were added to each well. The cytotoxicity assay and the determination of cytotoxic activity were carried out exactly as described above.

Results

Analysis of Cell Proliferation in the AMLR. Cell proliferation, as measured by [^{3}H]-thymidine incorporation, and the viability of E^+ cells cultured with autologous mitomycin-treated E^- cells was compared with unstimulated E^+ cells. Cell proliferation was demonstrable by day 3, and the peak [^{3}H]thymidine incorporation occurred on day 7. The maximum number of viable cells recovered from the AMLR cultures varied from donor to donor between the 5th and 9th d. Almost all the MMC-treated E^- cells were dead after 3 d in culture (Table II).

The surface markers of the cells generated after 7 d in AMLR culture are compared with the characteristics of the input cells in Table III. Most of the cells stained with the MAb 9.6. There were modest changes in the $OKT4^+$ and $OKT8^+$ populations. Cells bearing Ia antigen and the 4F2 marker were significantly increased. In contrast,

TABLE II

Percent Survival of Cultured Cells in AMLR

Days in culture	AMLR culture	Control culture	
	$E^+ + E^-_{MMC}$*	E^+ alone	E^-_{MMC} alone§
1	65.5 ± 6.0	47.5 ± 18.6	35
3	77.3 ± 4.9	49.7 ± 18.0	15
5	83.4 ± 23.1	47.7 ± 17.0	5
7	83.2 ± 31.3	48.0 ± 19.1	0
9	110.9 ± 63.2	52.5 ± 14.3	0
12	94.1 ± 51.2	40.5 ± 19.5	ND‡
20	38.1§	7.1§	ND

* E^+ cells were cultured with mitomycin C-treated autologous E^- cells.
‡ Not Done.
§ Mean of two experiments. Others were mean ± SD of 12 experiments.

TABLE III
Cell Populations Generated in AMLR Culture

Staining with MAb	Before culture		After 7 d culture	
	E^+*	E^-	E^+	$E^+ + E^-_{MMC}$‡
9.6	84.0 ± 8.4	14.4 ± 6.3	92.7 ± 6.0	95.8 ± 1.6
OKT_4	52.3 ± 7.6	8.3 ± 3.8	67.2 ± 7.3	66.8 ± 7.3
OKT_6§	0	0	0	0
OKT_8	31.0 ± 8.8	6.0 ± 3.2	25.4 ± 5.1	25.6 ± 7.5
OKT_9§	0	0	0	1.7 ± 1.7
OKT_{10}§	0	0	0	8.7 ± 3.7
OKI_{a1}	11.5 ± 7.4	73.8 ± 2.9	3.5 ± 2.9	39.2 ± 17.4
$4F_2$	4.7 ± 4.0	45.5 ± 13.5	8.4 ± 6.6	68.3 ± 11.1
OKM_1	19.2 ± 6.1	46.5 ± 9.3	4.0 ± 2.9	4.4 ± 3.6
Leu-7	22.7 ± 6.6	7.0 ± 6.9	7.9 ± 3.7	4.4 ± 2.3
Agg IgG‖	20.3 ± 6.7	60.0 ± 10.3	4.5 ± 1.2	4.3 ± 3.9

* E^+ cells.
‡ E^- cells treated with mitomycin C were used as stimulators in AMLR culture and no E^- cells survived after 7 d culture.
§ Mean ± SD of three experiments. Others were of 12 experiments.
‖ Detection of IgG Fc receptor positive cells by aggregated IgG.

there were significant reductions in the $OKM1^+$ cells, Leu-7^+ cells, and cells with Fc receptors. Small numbers of $OKT9^+$ and $OKT10^+$ cells that were not apparent before culture could be demonstrated after 7 d. In all the AMLR cultures, the percent of $4F2^+$ cells was always much higher than that of $OKIa1^+$ cells.

Generation of AMLR Cytotoxic Cells. As previously reported, AMLR killer cells develop when cultured in fresh AP or serum (16). Aged AP or serum (1 mo or 3 mo stored at −20°C), fresh heterologous plasma or serum from donors with similar or different blood types, and fetal calf serum all were much less supportive of cytotoxic cell production (50% or greater reduction in ^{51}Cr release from target cells).

The kinetics of AMLR-generated killer cell activity against three different cell lines at a 20:1 E/T ratio are shown in Fig. 1. Cells able to lyse K562, Daudi, and Raji targets generally appeared on day 5 and were maximally cytolytic on day 7 or 9 of culture. K562 cells appeared to be the most susceptible targets for AMLR killing; Raji cells were least sensitive (Fig. 2).

The effect on cytotoxicity of adding increasing concentrations of aggregated IgG to E^+ cells at various times during the AMLR culture is shown in Fig. 3. On day 0 (the E^+ obtained through overnight E rosetting), the lysis of K562 targets was reduced significantly by 30 and 300 μg of aggregated IgG. A similar effect was seen on day 3, but by day 7 the AMLR killer activity was influenced only slightly by the addition of aggregated IgG. These findings suggest that most of the cytotoxicity is exerted by Fc^- cells, with a small participation from the 4–5% of Fc^+ cells that survive cultures for 1 wk (Table III).

Characterization of the AMLR Cytotoxic Cell with MAb. The cytotoxic cells generated in a 7-d AMLR culture were treated with a variety of MAb plus C′, and their susceptibility was compared with fresh NK activity against a K562 cell line. Conventional NK activity was significantly reduced only by treatment with MAb 9.6, OKM1, and Leu-7 (Fig. 4). C′ lysis with MAb to the cell surface antigens of activated T lymphocytes (4F2 and OKIa1) caused only slight reduction. In contrast to the NK

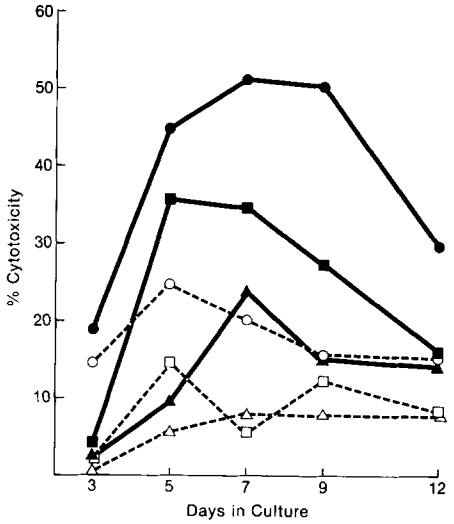

FIG. 1. A kinetic analysis of AMLR killer cell activity against three cell lines. E^+ cells were removed from AMLR cultures at the times indicated and tested for their cytotoxicity at an E/T ratio of 20:1 against targets K562 (●), Daudi (■), and Raji (▲). The percent cytotoxicity by the cells from stimulated cultures (solid lines) were compared with T cells cultured without stimulators (broken lines).

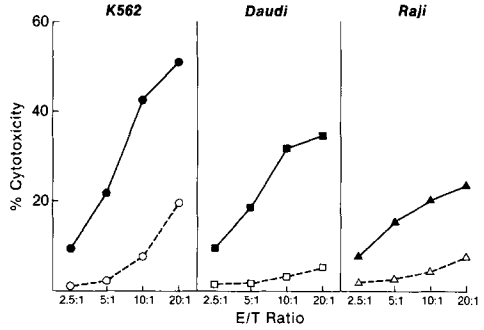

FIG. 2. A comparison of the cytotoxicity of cells removed from AMLR culture on day 7 against targets K562 (●), Daudi (■), and Raji (▲). Various E/T ratios were examined in a 4-h chromium-release assay. E^+ cells that had been stimulated by non-T cells (solid lines) were compared with non-stimulated E^+ cells (broken lines).

activity, the killing generated in the 7-d AMLR cultures was only eliminated by MAb 9.6 or 4F2 plus C′ treatment. NK-specific MAb OKM1 and Leu-7 did not reduce the AMLR killing significantly (Fig. 5). There was slight, but probably significant reduction of AMLR cytotoxic activity against the K562 cell line by treatment with anti-Ia plus C′. A strong correlation could be demonstrated between the percentage of cells in a 7-d AMLR culture that stained with the MAb 4F2 and the percent lysis of K562 targets ($\gamma = 0.87$, $P < 0.002$) (Fig. 6). Of particular interest was the finding that MAb specific for cytotoxic T cells (OKT8 and 9.3) had no inhibitory effect on either NK or AMLR-generated cytotoxicity. In fact, in most cases, these antibodies increased both types of killing. C′-mediated lysis with OKT4 antibody always enhanced AMLR killing presumably because the removal of the large number of $OKT4^+$ cells increased the proportion of the cytotoxic effectors in the residual cell

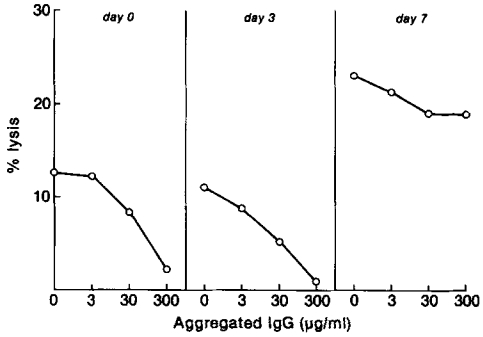

FIG. 3. The effect of the addition of varying concentrations of aggregated IgG on cytotoxicity of cells taken at different times from AMLR culture. A 4-h chromium-release assay with K562 targets at an E/T ratio of 20:1 was performed with E^+ cells separated after overnight incubation (day 0) and from AMLR cultures on days 3 and 7. The percent lysis (○) represents the difference between chromium released by stimulated cells minus that released by the unstimulated cells.

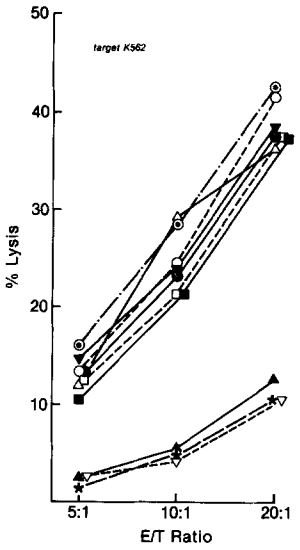

FIG. 4. NK cell activity against K562 targets after treatment with MAb plus C′. C′ alone (○), OKT4 + C′ (●), OKT8 + C′ (△), 9.3 + C′ (⊙), 4F2 + C′ (□), OKI_{a1} + C′ (■), T101 + C′ (▼), Leu-7 + C′ (*), 9.6 + C′ (▽), and OKM_1 + C′ (▲).

population. Results similar to those shown in Fig. 5 were seen in four separate experiments and also when Raji cells were used as targets.

The susceptibility of fresh NK- and AMLR-generated cytotoxic cells to blockade by MAb was assayed by adding 1.5 μg/ml (final concentration) of each individual antibody in the absence of C′. With the exception of a slight amount of blocking by MAb 9.6, there was no significant effect from the addition of any MAb to either cytotoxic effector cell assay.

Relative Importance of $Ia1^+$- and $4F2^+$-bearing Cells as AMLR Killers. The role of cells with an $Ia1^+$ or $4F2^+$ phenotype was studied further by establishing AMLR cultures for 7 d, treating the remaining cell population with MAb plus C′ or C′ alone, then pulsing with [^{3}H]thymidine and harvesting 18 h later. The cytotoxicity of the residual cells was determined using K562 as targets. The relationship between AMLR killer

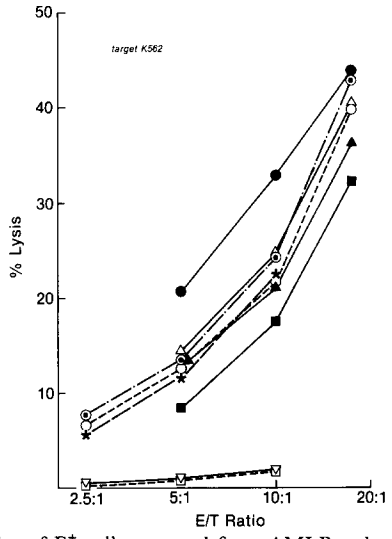

FIG. 5. The cytotoxic activity of E^+ cells removed from AMLR culture at 7 d on K562 targets after treatment with MAb and C′. See Fig. 4 for the designations of C′ alone or with the individual MAb.

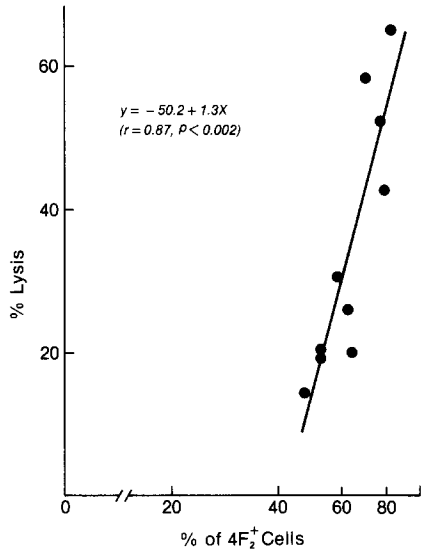

FIG. 6. Correlation between the percentage of cells in a 7-d AMLR culture that stained with the MAb 4F2 and the percent lysis (uncorrected) of K562 targets (E/T, 10:1).

activity and cell proliferation is demonstrated in a typical experiment (shown in Table IV). Treatment with OKIa1 plus C′ reduced [^{3}H]thymidine incorporation by ~35% and cytotoxicity by a similar amount. In contrast, 4F2 plus C′ treatment completely eliminated both thymidine incorporation and AMLR-induced killing. These results suggest that the majority of the cytotoxicity and most of the DNA proliferation seen after 1 wk in culture is from $4F2^+$ cells. The role of the cells bearing the surface Ia antigen was studied further, since both 4F2 and OKIa1 are known to be expressed on proliferating or activated T cells. When cells taken after 7 d in AMLR

TABLE IV
Relationship Between AMLR Killer Activity and Cell Proliferation

	Treatment		
	C′ alone	C′ + OKI_{a1}	C′ + $4F_2$
^3H incorporation*	38,390 ± 22	24,086 ± 834	1,128 ± 150
AMLR killer activity‡	30.3	19.8	1.8

* [^{3}H]thymidine incorporation was expressed as CPM/2 × 10^5 cells.
‡ AMLR killer activity (percent lysis) against K562 cell line was assessed at E/T ratio, 10:1.

culture were treated with the respective MAb plus C′, and then stained, it could be shown that 30% of the cells surviving after OKIa1 plus C′ treatment were $4F2^+$, but that no $OKIa1^+$ cells remained after 4F2 plus C′ treatment. We would conclude, therefore, that AMLR killers are heterogeneous for the Ia1 phenotype; a portion are positive but the majority are not.

Discussion

Despite reports to the contrary, cytotoxic effector cells can be generated in the AMLR. Their appearance parallels the DNA synthetic response of the stimulated T cell population, but persists for several days longer, as shown by Tomonari (16) and in this paper. AMLR killers lyse a variety of targets, including human lymphoid cells of both the B and T cell class, autologous B cell lines transformed by Epstein-Barr virus, autologous and allogeneic mitogen blasts, and some xenogenic cell lines (16, 17).

The reason why some investigators failed to detect AMLR-generated cytotoxic cells is not clear. Tomonari (16) ascribed his success to the use of AP in the AMLR culture medium. Our findings are confirmatory; namely that fresh human plasma or serum or fetal calf serum are equally supportive of the proliferative response (detected by [^{3}H]thymidine uptake) in the AMLR cultures, but that efficient cytotoxicity was dependent on the use of fresh human plasma (or serum). An autologous source was better than heterologous plasma and the blood group status of the donor seemed to have no influence. The contribution of AP to the generation of AMLR killer activity requires further clarification, but in a preliminary report, Hausman et al. (47) have observed that serum from major histocompatibility complex-identical or haplo-identical individuals is as effective as AP.

Because of a number of similarities, Tomonari (16) suggested that the auto-MLR cytotoxic effector was in the NK lineage. There are important differences, however, including the fact that his cytotoxic cells lysed autologous B cell lines and Daudi cells, which are usually insensitive in short-term cytotoxicity assays to NK cells, and the observation that the AMLR killers are not Fc receptor-bearing cells (17). Because of these discrepancies, it was considered of interest to define further the AMLR cytotoxic effector cell with the use of MAb.

Of the 12 MAb chosen, 9 react with distinct T cell differentiation antigens (9.6, 9.3, T101, OKT4, OKT6, OKT8, OKT9, OKT10, and 4F2), 1 recognizes a human Ia antigen (OKIa1), 1 the NK and monocyte lineage (OKM1), and 2 (Leu-7 and OKM1) human NK cells. Most fresh NK cells express antigens recognized by MAb 9.6, OKM1, and Leu-7; C′-mediated lysis with these antibodies eliminated most of

the cytotoxicity of PBL tested before establishment of the AMLR cultures (Fig. 3). This is similar to the findings of Zarling et al. (48, 49) and Fast et al. (28) who reported that C′-mediated lysis with OKM1, 9.6, or OKT11A (a 9.6 equivalent) efficiently abrogated fresh NK activity. In contrast, the AMLR cytotoxic effectors were only eliminated by treatment with MAb 4F2 or 9.6 and C′ and reduced by Ia1$^+$ antibody treatment. Similar results were obtained with both K562 and the Raji cells as targets. The cytotoxic cell is most likely from the T lymphocyte lineage, because >95% of the cells present at the end of 1 wk in the AMLR culture stain with MAb 9.6, which recognizes a 50,000 dalton molecule on the surface of thymocytes and T cells and because MAb 9.6 plus C′ treatment abrogated most of the AMLR killer activity.

Fresh NK cells show a variable expression of density and avidity of Fc gamma receptors (50). Most of the fresh NK activity can be blocked by prior exposure to aggregated IgG (18, 20). In contrast, only a slight to moderate suppression of the activity in AMLR killers by aggregated IgG was found in our experiments at the end of 1 wk in culture. This is consistent with our previously reported observation that the removal of Fc gamma-positive cells by adherence to immune complexes could not deplete AMLR killer activity (17). In a few experiments, a slight decrease in AMLR killer activity was noted after treatment with NK-specific MAb and C′ or with aggregated IgG, so a small part of the killing may be attributable to surviving NK cells. This is consistent with the observation that 5–10% of the cells remaining at the end of 1 wk in the AMLR and control cultures were OKM1$^+$, Leu-7$^+$, and Fc receptor positive (Table III).

The AMLR cytotoxic effector cell appears to be distinct from the classical CTL which developed during allosensitization. The latter are OKM1$^-$, OKT3$^+$, and OKT8$^+$, and displayed a cell surface activation antigen 4F2, but probably not Ia (51). ~25% of the cells present after 1 wk in the AMLR cultures stained with the OKT8 MAb, but, surprisingly, treatment of the culture with this reagent plus C′ enhanced rather than ablated the cytotoxicity against the K562 target. Further support for the separation between CTL and AMLR killer cells comes from the finding that MAb OKT8$^+$ and 9.3, which normally block CTL (in the absence of C′) (28, 52, 53), had no effect on AMLR killer activity.

A source of confusion in the past stems from the fact that at least two cytotoxic populations develop during the course of a MLR. The first, the classic CTL, kills the allogeneic target, whereas the other lyses a variety of cultured cell lines and is independent of histocompatibility relationships (16). The latter appears several days earlier in culture and the two can be distinguished by cross cold target inhibition (54, 55). Their coexistence may account for the discrepant results obtained using MAb and C′ to define the phenotype of the responsible cells. In such studies, diminution without complete elimination of cytotoxic activity is usually ascribed to the lesser sensitivity of this method as compared with cell sorting or the fact that the cells with fewer surface antigens escape the C′-mediated effects (28). Alternatively, the observed cytotoxicity can be caused by two cell populations with different phenotypes. Such mixtures might explain why, for instance, Grimm et al. (56) could not totally eliminate CTL activity produced in the allo-MLR with C′-mediated lysis using OKT3 and OKT8 antibodies.

The cells that the AMLR killers most closely resemble are the nonspecific cytotoxic

cells variously described as NK-like, anomalous killers, or lymphokine-activated killer cells (LAK) (56). Typically, this class of cytotoxic cells is comprised of T lymphocytes that have a broad range of target specificities, lack Fc receptors, and show a limited number of characteristic cell surface antigens. They all appear to express the 4F2 phenotype (Table V).

The relationship of the various cytotoxic effector cells; i.e., conventional NK, induced NK-like, and allospecific CTL, to one another is unresolved. One suggestion is that they represent different stages in the development or maturation of a single lineage. The finding that the AMLR killers display activation antigens, but not OKT8, could reflect the lack of a differentiation signal provided by an allospecific stimulus. We favor an alternative view; namely, that the two are distinct T cell populations. Two lines of evidence can be cited. First, MLC-generated CTL and NK-like cells have separate target specificities when analyzed in a single cell binding and cytotoxicity assay (54). Second, the subset of T lymphocytes that respond to autologous stimulation is separable from alloreactive T cells (55, 57).

According to MacDonald and Lees (58) cytotoxic T cell activity can be induced in the allogeneic MLR independently of DNA synthesis. On the other hand, DNA synthesis appears to be required for the generation of AMLR killing, as reported by others (16), and in this paper (Table V). The peaks for each response were dissociated, and no clear-cut correlation was observed between [^{3}H]thymidine uptake and AMLR killer activity. Blast transformation, DNA synthesis, and cell proliferation are not synonymous, however, and instances have been reported where [^{3}H]thymidine incorporation has occurred without demonstrable cell division (59). Grimm (56) has suggested that the LAK system is probably the common pathway that explains most of the nonclassic cytotoxic cells. If she is correct, and nonspecific cytotoxicity, anomalous killers, and other unusual forms of killer cells are the result of activation of precursor T lymphocytes by interleukin 2 (IL-2), then proliferation would appear to be common to all of them. IL-2 is unquestionably made in the AMLR, and cellular proliferation appears to be directly related to its production (9, 11, 12). From studies in progress, however, we would question whether IL-2-stimulated proliferation alone

TABLE V

Characteristics of Cells with Cytotoxic Capacity

Cell type	Cell surface phenotype						Day of peak activity	Sensitivity against mitogen-stimulated autologous cells	Requirement of proliferation for the generation of killer cells	Reference
	IgG Fc	pan T (Leu-1, OKT_3, T_{101}, 9.6)	CTL (OKT8, 9.3)	Ia (OKI_{a1})	NK (OKM1, Leu-7)	Activated T ($4F_2$)				
NK	+*	+	−‡	−	+	−	0	−	−	18–20, 28, 41, 43, 48–50§
CTL	−	+	+	−‖	−	+	5–7	−	+‖	21–22, 28, 37, 40, 48, 51–52, 58, 60
LAK	NR¶	+	+	NR	−	+	5–7	−	+	56
AMLR Killer	−	+	−	−	−	+	7–9	+	+	13, 15–17§

* Usually present.

‡ Usually absent.

§ Discussed in this paper.

‖ Conflicting data were also reported.

¶ Not reported.

is sufficient for the production of auto-MLR killers, since cells from patients with certain diseases (rheumatoid arthritis as an example) give a near normal thymidine incorporation but produce very few cytotoxic cells at the end of seven days in culture (manuscript in preparation).

Summary

Cytotoxic cells are produced in an autologous mixed leukocyte reaction (AMLR). At 1 wk in culture the AMLR killers are mainly IgG Fc^- cells and can kill autologous lymphoblastoid cell lines and Raji and Daudi targets that are usually resistant to natural killer cell (NK) lysis. To define the phenotype of these cells, we have used complement (C′)-mediated lysis with monoclonal antibodies (MAb). AMLR killer activity was virtually eliminated by treatment with C′ and 9.6 or 4F2, but the cytotoxic cells did not express NK-specific antigens, OKM1 and Leu-7, nor cytolytic T lymphocyte-specific antigens, 9.3 and OKT8. None of the 10 MAb used could significantly block cytotoxicity at the final concentration of 1.5 mcg/ml which is generally sufficient to inhibit CTL. The majority of cells at 1 wk in AMLR cultures stained with T cell activation antigens Ia and 4F2; AMLR killing was proportional to the percentage of $4F2^+$ cells but unrelated to the expression of Ia antigen.

The authors wish to thank Ms. Leslye Rucker for her excellent secretarial assistance, Doug Redelman for useful discussion and review of the manuscript, and Anthony Fauci for generous supplies of the 4F2 monoclonal antibody.

Received for publication 9 December 1982.

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