IN VITRO GENERATION OF TUMOR-SPECIFIC CYTOTOXIC LYMPHOCYTES

Secondary Allogeneic Mixed Tumor Lymphocyte Culture of Normal Murine Spleen Cells*

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The mixed tumor lymphocyte culture (MTLC)[1] has been reported as being an effective in vitro means of generating lymphocytes cytotoxic to leukemia cells (1). The cytotoxic capabilities of cells harvested from MTLC reactions have been demonstrated in lymphocyte-mediated cytolysis (LMC) assays (2), and the reactivities of these lymphocytes have corresponded with in vitro cytotoxicities of lymphocytes harvested from tumor-immune animals (3) as well as with in vivo transplantation resistance (4). However, the MTLC-LMC system has only corresponded with tumor immunity studies of highly antigenic systems. MTLC have succeeded in generating cytotoxic lymphocytes from normal lymphoid populations (5) and have enhanced the cytotoxicity of cells harvested from tumor-immune animals (6). This has only been possible when the tumor cell used was capable of generating an eventually regressing tumor in vivo. Lymphocytes harvested from mice with progressively growing tumors have been incapable of being stimulated via MTLC to display in vitro cytotoxicity against syngeneic tumor cell targets (5, 6).

Early investigation showed that leukemias induced by different strains of murine leukemia virus (MuLV) were cross-reactive in their ability to confer syngeneic tumor transplantation resistance (7). Virus-induced leukemia cells were shown to share serologically defined cross-reactive antigens with allogeneic leukemia cells induced by the same virus (8). Furthermore, immunization with allogeneic leukemia cells was often more effective in conferring syngeneic tumor transplantation immunity than was immunization with syngeneic tumor cells (9). Because in many cases, high immunizing concentrations of allogeneic leukemia cells were utilized, allogeneic immunization might have evoked a

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[1] *Abbreviations used in this paper:* Con A, Concanavalin A; cpm, counts per minute; FCS, fetal calf serum; FLV, Friend leukemia virus; g, glycoprotein; [^{3}H]TTP, tritium-labeled thymidine-5′-triphosphate; LMC, lymphocyte-mediated cytolysis; LU, lytic unit; MLC, mixed lymphocyte culture; MTLC, mixed tumor lymphocyte culture; MuLV, murine leukemia virus; p, protein; RLV, Rauscher leukemia virus; RT, reverse transcriptase; TASA, tumor-associated cell surface antigen.

more effective immunity because allogeneic cells presented a greater tumor antigen load or because they displayed histoincompatible antigens which provided an allogeneic effect.

Transplantation resistance demonstrated in these early studies was always conferred by immunization with syngeneic or allogeneic cells which actively produced infectious MuLV. More recently, virus-producing tumor cells have been shown to express viral structural proteins and glycoprotein (10) at the cell surface. These viral polypeptides are capable of evoking a detectable immune response in heterologous or homologous species (11, 12). Viral antigens may be partially responsible for the anti-tumor immunity demonstrated in in vivo transplantation resistance experiments to virus-producing cells.

Because human leukemia cells have not been demonstrated to be effective producers of C-type RNA tumor virus nor to express viral proteins at the cell surface, we have focused our attention on nonvirus producing tumor cells (nonproducers). Recent studies have concluded that nonproducers are more tumorigenic and less antigenic than virus-producing leukemia cell lines (13) and attempts to demonstrate in vivo tumor-specific transplantation resistance to nonproducers have been difficult (14).

Since allogeneic leukemia cells were effective generators of in vivo syngeneic tumor immunity, we have attempted to use allogeneic nonproducer leukemia cells in vitro to generate tumor-specific cytotoxic lymphocytes. This report describes how repetitive MTLC stimulation of normal murine spleen cells with allogeneic nonproducer leukemia cells, produces a subpopulation of lymphocytes with syngeneic tumor-specific cytotoxicity. The antigen reactivity of these cytotoxic lymphocytes may be directed against tumor-associated cell surface antigens (TASA) not necessarily related to viral antigens.

Materials and Methods

Mice. Age-matched adult females, C57BL/6 and DBA/2, 5- to 7-wk-old were used in immunization experiments. Adult females from each strain (5- to 10-wk-old) were used as sources of normal spleen, lymph node, and thymus cells. All mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Tumor Cells. Cells used for immunization and in MTLC were F4-5, a nonvirus producing subclone of F-4, syngeneic to the DBA/2 mouse, (obtained from Dr. W. Ostertag of the Max Planck Institute, Göttingen, Germany), and FBL-3(Hn) a nonvirus-producing cell line syngeneic to the C57BL/6. Both cell lines were originally induced by Friend leukemia virus (FLV). FBL-3(Hn) was derived from the FBL-3 leukemia cell (originally obtained from Dr. Ronald Herberman of the National Cancer Institute) by adaptation of ascites-passaged FBL-3 cells to long-term in vitro culture in our laboratory. Unlike FBL-3, FBL-3(Hn) does not generate a regressing tumor after subcutaneous inoculation in syngeneic mice. In fact, subcutaneous injection of as few as 10^2 live FBL-3(Hn) cells produces a progressively growing, lethal tumor. Tumor cells used as targets in cytotoxicity assays included FBL-3(Hn), F4-5, the P815 mastocytoma cell (obtained from Dr. H. R. MacDonald, Ontario Cancer Research Institute, London, Ontario, Canada), and the FLV-induced FLD-3 (obtained from Dr. W. Ostertag) both syngeneic to the DBA/2. Other tumor targets included the Rauscher leukemia virus (RLV)-induced RBL-5 and the chemically-induced EL-4 (Gross virus negative) both syngeneic to the C57BL/6 mouse (both obtained from Dr. R. Herberman). All cells were maintained in continuous culture in vitro at 37°C in a humidified atmosphere of 5% CO_2 in air.

Immunization. Mice were immunized subcutaneously in the right flank with 5×10^6 mitomycin-treated (15) (100 μg/ml) allogeneic nonproducer leukemia cells once a week for a period of 6 wk. Spleen cells were harvested for MTLC 2–4 wk after completion of the immunization schedule.

Assays for Viral Characterization of Tumor Cells. All tumor cells were subjected to the following assays:

X-C ASSAY FOR PRODUCTION OF MATURE MuLV. The method used was exactly as previously described (16) with the exception that the SC-1 cell line (17) was used as the primary cell inoculated by virus. Samples for inoculation were prepared by sampling 24 h tissue culture medium from tumor cells in their exponential growth phase. By inoculating multiple tissue culture cluster wells (no. 3506, Costar, Data Packing Corp., Cambridge, Mass.) with $\log_2$ or $\log_{10}$ dilutions of the original tumor cell culture supernate, the amount of infectious MuLV produced was quantitated in terms of plaque-forming units/milliliter of tumor cell tissue culture supernate.

ASSAY FOR REVERSE TRANSCRIPTASE (RT) ACTIVITY OF TUMOR TISSUE CULTURE MEDIA. Tissue culture medium exposed for 24 h to tumor cells in their exponential growth phase was harvested for testing. The RT activity assay was conducted as previously described (18). A positive test for RT activity was indicated by high [^{3}H]thymidine-5′-triphosphate ([^{3}H]TTP) incorporation into the oligo dT poly rA primer template aliquot as opposed to [^{3}H]TTP incorporation into the oligo dT poly dA primer template aliquot. Positive control tests were conducted by using no. 8203-01 RNA directed DNA polymerase purified from RLV peak fraction (Litton Bionetics, Bethesda, Md.).

COMPLEMENT-MEDIATED ANTIBODY-DEPENDENT CYTOLYSIS. Tumor cells used as targets in LMC (Cr-relase) assays were tested for the presence of cell surface viral proteins by using antisera obtained from Dr. Jack Gruber (Office of Program Resources and Logistics, Viral Oncology, National Cancer Institute, Bethesda, Md.). Absorbed goat antisera to gp-71, p-30, and p-12 (RLV) were tested. Tumor cells were labeled with 250 μCi of $Na_2\ ^{51}CrO_4$ (sp act 100–400 mCi/mg Cr), (Amersham/Searle Corp., Arlington Heights, Ill.). The target cell concentration was adjusted in RPMI 1640 (Grand Island Biological Co., Grand Island, N.Y.) with 10% fetal calf serum (FCS) to 0.1 counts per minute (cpm)/cell. Target cells were distributed in multiple 100-μl volumes to individual wells of V-bottom microplates (no. 1S-MVC-96-TC, Linbro Chemical Co., New Haven, Conn.). Log_2 dilutions of each antisera were added in 50-μl aliquots to multiple microplate wells containing the target cell suspension. After a 30-min, 37°C incubation, 50 μl of absorbed (19) rabbit complement (Gibco Diagnostics, The Mogul Corp., Chagrin Fall, Ohio) was added to each well containing either medium, ZAP Isoton Solution ([three drops added to 5 ml of double distilled water], [Coulter Electronics Inc., Hialeah, Fla.]), or a dilution of specific antisera and the reaction was allowed to continue for an additional 90 min incubation at 37°C. Chromium release was terminated by a 400 *g* centrifugation at 4°C. Supernate (100 μl) was removed and counted in 3.5 ml of Biofluor Scintillant Fluid (NEF 961, New England Nuclear, Boston, Mass.) by a liquid scintillation counter. Percent cytotoxicity was calculated according to the following equation.

$$\text{Percent cytotoxicity} = \frac{\text{cpm experimental} - \text{cpm complement} + \text{target cell control}}{\text{cpm maximum} - \text{cpm complement} + \text{target cell control}} \times 100$$

Target cell surface density of particular viral antigens was expressed by the highest titer of specific antisera necessary to cause 20% complement-mediated antibody-dependent lysis. The absorbed complement rarely caused over 5% spontaneous lysis of the target cells.

In Vitro Generation of Cytotoxic Lymphocytes. Spleen cells from immunized and/or normal mice were selected for use as responding cells in several in vitro culture treatments. Cells were teased apart from the intact organ with forceps, minced, and passed through successively smaller gage needles to ensure single cell suspensions. Erythrocytes were removed from the cell population by hypo-osmotic shock, and remaining cells were counted with an electronic Coulter Counter (Coulter Electronics). After determining viability via trypan blue exclusion, cells were diluted in Click's medium (20) (Altick Associates, Hudson, Wis.) supplemented with 300 μg/ml fresh L-glutamine (Gibco Diagnostics), 50 U/ml penicillin, 50 μg/ml streptomycin, 16 μmol/ml $NaHCO_3$, 25 μmol/ml HEPES buffer (Gibco Diagnostics), and 2% heat inactivated FCS. Spleen cell populations were adjusted to a final concentration of 2.5×10^6 cells/ml.

Primary MTLC stimulation was conducted by mixing 10 ml of spleen cells with 10 ml of mitomycin-treated (100 μg/ml) nonproducer murine leukemia cells which had been adjusted in supplemented Click's medium to a concentration of 6.25×10^4 cells/ml. Primary MTLC were cultured upright in 30-ml tissue culture flasks (no. 3012, Falcon Plastics, Division of BioQuest, Oxnard, Calif.) in an humidified atmosphere of 5% CO_2 in air, at 37°C. Identical culture conditions were used for primary mixed lymphocyte cultures (MLC) except that a 1/1 ratio of responder to stimulator lymphocytes was used and the stimulatory spleen cell population was prevented from

proliferating by a 1,500 rad X irradiation (Cobalt source). Primary MTLC (MLC) were harvested after 5 days in culture and viable cells were used as effector cells in LMC assays or subjected to secondary stimulation.

Secondary MTLC stimulations were conducted by resuspending responding lymphocytes from primary MTLC flasks in two 10-ml aliquots of fresh supplemented Click's medium and mixing each aliquot with 10 ml of mitomycin-treated nonproducer leukemia cells (6.25×10^4 cells/ml). Similarly, secondary MLC stimulation was achieved by harvesting viable cells from primary MLC flasks, and resuspending them in 20 ml of fresh supplemented Click's medium. This suspension was placed in new flasks (10 ml/flask) with 10 ml of X irradiated (1,500 rad) allogeneic spleen cells (2.5×10^6 cells/ml). Secondary Concanavalin A ([Con A], [Miles-Yeda Laboratories, Rehoveth, Israel]) stimulation of either MTLC or MLC reactions was conducted by resuspending viable cells harvested from primary MTLC (MLC) in 20 ml of fresh supplemented Click's medium and adding to each 10 ml aliquot another 10 ml fresh supplemented Click's medium containing Con A (5 μg/ml).

All secondary stimulations (MLC, MTLC, or Con A) were conducted upright in 30-ml tissue culture flasks in an humidified atmosphere of 5% CO_2 in air at 37°C. Secondary stimulation cultures were terminated two days after their initiation and viable lymphocytes harvested for use as effector cells in LMC assays.

LMC Assay. Target cells were labeled as described above and distributed in 100-μl volumes to multiple V-bottom microplate wells. Effector cells were harvested from 5 day primary MTLC (MLC) or 2 day secondary MTLC (MLC) and adjusted to give an effector/target cell ratio of at least 50/1. Log_2 dilutions of the effector cells were made and added to the targets in 100-μl volumes. Each effector/target cell ratio was repeated in replicate microplate wells. Microplates were then centrifuged at 350 *g* for 10 min and incubated in 5% CO_2 at 37°C for hours. The ^{51}Cr release reaction was stopped by a 400 *g* centrifugation for 10 min at 4°C. A 100-μl supernatant aliquot was removed from each well, suspended in 3.5 ml of Biofluor Scintillant Fluid, and counted on a liquid scintillation counter. cpm of chromium release from individual medium, ZAP (maximum), and effector/target cell multiplicates were averaged, and standard deviation and percent specific lysis were calculated with the aid of a basic computer program and the Dartmouth Time Sharing System. Percent specific lysis was determined by using the following equation:

$$\text{Percent specific lysis} = 100 \times \frac{\text{experimental cpm} - \text{medium control cpm}}{\text{maximum release cpm} - \text{medium control cpm}}$$

Negative values were interpreted by the computer as 0% specific lysis. 1 lytic unit (LU) was defined as the number of effector cells required to cause 30% specific lysis. Data is displayed both graphically and in tabular form as percent cytotoxicity observed at an effector/target cell ratio of 100/1, and as LU/culture. In cases where 30% cytotoxicity was not achieved even at relatively high effector/target cell ratios, LU were determined by graphical interpolation.

Results

Virological Characterizations of Hemopoietic Tumor Cell Lines. The results of tests for virus production and cell surface viral antigen expression of the tumor cells used, appear in Table I. Tumor cells, syngeneic to the C57BL/6 mouse included the RLV-induced RBL-5, which was confirmed in X-C and R-T assays as a low titer virus producer and which demonstrated cell membrane expression of gp-71. FBL-3(Hn) tested negative for MuLV production in X-C and R-T assays yet expressed gp-71 at the cell surface. Supernates harvested from cultures of EL-4 (also syngeneic to the C57BL/6), were negative for virus production and the cells themselves were void of cell surface expression of viral antigens gp-71, p-30, or p-12. Cells tested in the DBA/2 histocompatibility model system included a confirmed virus producing line, FLD-3, whose cell membranes were positive for expression of gp-71 and p-30. The FLV-induced nonproducer, F4-5 was also positive for gp-71. The P815 tumor cell tested negative for

TABLE I
Viral Characterizations of Tumor Cells

Cell designation	Host mouse	Transforming agent	RT activity*	MuLV Production‡	Cell surface expression of viral antigens‖		
					GP-71	p-30	p-12
RBL-5	C57BL/6	RLV	9,606/5,439	57.2	1/125	§	§
FBL-3(Hn)	C57BL/6	FLV	1,606/1,502	0.0	1/640	§	§
EL-4	C57BL/6	Benzanthracene	3,560/9,430	0.0	§	§	§
FLD-3	DBA/2	FLV	387,454/16,988	1,818.0	1/320	1/320	§
F4-5	DBA/2	FLV	1,392/5,665	0.0	1/50	§	§
P815	DBA/2	Methylcholanthrene	57,575/52,893	0.0	§	§	§
Purified RLV RT			58,139/6,570				

* Expressed in terms of cpm incorporation of [^{3}H]thymidine-triphosphate by oligo dT poly rA template aliquot/cpm incorporation of [^{3}H]thymidine-triphosphate by oligo dT poly dA template aliquot.

‡ Expressed in terms of plaque-forming units/milliliter of tissue culture supernate as determined by the X-C assay.

§ No cell surface expression of viral protein by complement-mediated antibody-dependent cytotoxicity, by using goat antisera produced against individual, purified RLV proteins.

‖ Expressed in terms of highest dilution of specific antisera yielding 20% cytotoxicity as measured by complement-mediated antibody-dependent-cytotoxicity. Goat antisera produced against RLV gp-71, p-30, and p-12.

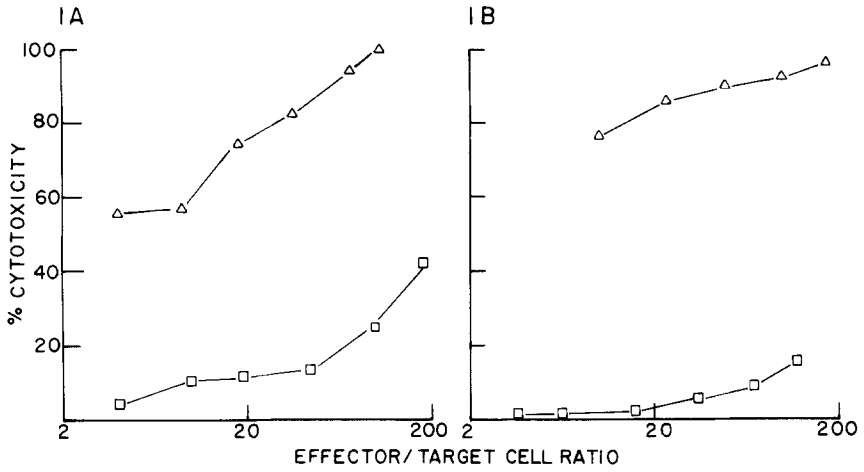

FIG. 1. (A) Lysis of the allogeneic tumor cell target F4-5 mediated by C57BL/6 lymphocytes subjected to a single allogeneic MTLC activation with F4-5 (□—□) and by C57BL/6 effector cells harvested after secondary allogeneic MTLC with F4-5 (△—△). (B) Lysis of the allogeneic tumor target cell FBL-3(Hn) effected by DBA/2 lymphocytes subjected to a single allogeneic MTLC activation with FBL-3(Hn) (□—□) and by DBA/2 lymphocytes harvested after secondary allogeneic MTLC activation with FBL-3(Hn) (△—△).

virus production by X-C and R-T assays and negative for cell surface expression of viral proteins gp-71, p-30 and p-12.

Secondary Allogeneic MTLC.

ELEVATION OF CYTOTOXICITY DIRECTED AGAINST ALLOGENEIC TUMOR TARGETS. Allogeneic tumor cell lysis by effector cells generated in primary and secondary allogeneic MTLC is displayed in Fig. 1. LMC directed toward the same allogeneic target cell which had been used as the MTLC stimulating agent dramatically increased when a secondary allogeneic MTLC activation was performed on cells surviving a primary allogeneic MTLC. This phenomenon was observed in both the C57BL/6 and DBA/2 model systems. Allogeneic cytotoxicity

TABLE II
Lysis of Syngeneic Tumor Cell Targets by MTLC Effector Cells

Source of spleen cells	Primary stimulation	Secondary stimulation	Syngeneic tumor cell cytotoxicity		
			FBL-3(Hn)	RBL-5	EL-4
C57BL/6	F4-5(A-MTLC)*	–	1% (6.7)	3% (11.2)	0% (0.0)
C57BL/6	F4-5(A-MTLC)	F4-5(A-MTLC)	31% (255.1)	37% (454.5)	18% (81.2)
C57BL/6	FBL-3(S-MTLC)‡	FBL-3(S-MTLC)	0% (0.0)	0% (0.0)	0% (0.0)
C57BL/6	DBA^x(A-MLC)§	–	3% (8.9)	5% (17.5)	0% (0.0)
C57BL/6	DBA^x(A-MLC)	DBA^x(A-MLC)	7% (16.7)	6% (12.5)	0% (0.0)
			FLD-3	F4-5	P815
DBA/2	FBL-3(A-MTLC)	–	0% (0.0)	0% (0.0)	0% (0.0)
DBA/2	FBL-3(A-MTLC)	FBL-3(A-MTLC)	43% (862.1)	41% (531.1)	38% (454.5)
DBA/2	F4-5(S-MTLC)	F4-5(S-MTLC)	1% (6.2)	0% (0.0)	3% (9.2)
DBA/2	C57BL/6^x(A-MLC)	–	3% (5.1)	0% (0.0)	1% (1.1)
DBA/2	C57BL/6^x(A-MLC)	C57BL/6^x(A-MLC)	5% (7.1)	0% (0.0)	7% (9.8)

* A, allogeneic.

‡ S, syngeneic.

§ DBA^x or C57BL/6^x refers to irradiated populations of respective spleen cells. Cytotoxicity is expressed in terms of lysis at an effector to target ratio of 100:1 and in LU per culture, e.g. 75% (5432.7).

demonstrated against F4-5 was 25% (208 LU/culture) at an effector/target cell ratio of 100/1 after primary allogeneic MTLC stimulation. Cytotoxicity rose to almost 100% (25,000 LU/culture) at a similar ratio, after a second allogeneic MTLC activation. DBA/2 spleen cells stimulated in primary allogeneic MTLC with FBL-3(Hn) generated lymphocytes only 13% cytotoxic to FBL-3(Hn) cells (55 LU/culture) at an effector/target cell ratio of 100/1. Spleen cells subjected to secondary allogeneic stimulation demonstrated 92% cytotoxicity (25,000 LU culture) at the same effector/target cell ratio. Similar high levels of allogeneic tumor cell lysis were also observed when lymphocytes from secondary allogeneic MTLC were tested against other allogeneic tumor cell targets (Table III).

DEMONSTRATION OF SYNGENEIC TUMOR CELL LYSIS. Cytotoxic cells capable of mediating syngeneic tumor cell lysis were generated only after a secondary allogeneic MTLC. This phenomenon was observed both in cases where C57BL/6 spleen cells were stimulated twice in vitro with allogeneic F4-5 tumor cells and when DBA/2 normal spleen cells were activated twice by FBL-3(Hn) cells. Syngeneic tumor cell lysis is represented both in terms of LU/culture and percentage of target cells lysed at an effector/target cell ratio of 100/1 (Table II). Several MTLC and MLC combinations were tested to determine if other in vitro stimulations would be as effective in generating lymphocytes capable of mediating syngeneic tumor cell lysis. Cells harvested from either a primary allogeneic MTLC or primary MLC were not cytotoxic to syngeneic tumor cells. Effector cells generated from secondary allogeneic MLC and secondary syngeneic MTLC (C57BL/6 spleen cells activated twice with mitomycin-treated FBL-3(Hn) cells and conversely DBA/2 spleen cells stimulated twice in vitro with F4-5 cells), did not demonstrate cytotoxicity directed against syngeneic tumor cell targets.

SPECIFICITY OF SYNGENEIC TUMOR CELL CYTOTOXICITY. After the observation

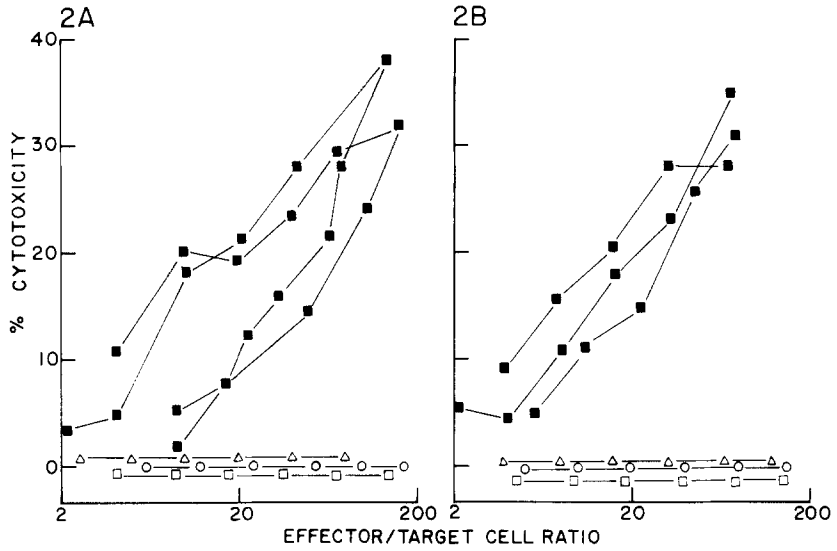

FIG. 2. (A) Lysis of the syngeneic tumor target cell FBL-3(Hn) (■—■) by C57BL/6 spleen cells harvested from secondary allogeneic MTLC stimulation with F4-5. Each line represents one of a number of repetitive experimental trials. Cytotoxicity mediated by these same effector cells against syngeneic normal lymph node (△—△), thymus (○—○), and Con A stimulated spleen cells (□—□) is also depicted. (B) Lysis of the syngeneic tumor cell F4-5 (■—■) demonstrated by DBA/2 lymphocytes harvested from secondary allogeneic MTLC activation with FBL-3(Hn). No cytotoxicity is mediated by these same effector cells against DBA/2 lymph node (△—△), thymus (○—○), and Con A stimulated spleen cells (□—□).

that secondary allogeneic MTLC stimulation of normal spleen cells led to proliferative generation of lymphocytes capable of effecting syngeneic tumor cell lysis, experiments were designed to test whether or not this lysis was directed solely against syngeneic tumor cell targets. Results of these experiments are represented graphically in Fig. 2. C57BL/6 spleen cells stimulated twice in allogeneic MTLC with F4-5 were cytotoxic to FBL-3(Hn) Friend leukemia cells yet were not cytotoxic to either syngeneic normal lymph node, thymus, or spleen cells stimulated 2 days previously with Con A (2.5 μg/ml). DBA/2 lymphocytes generated in secondary allogeneic MTLC with FBL-3(Hn) were highly cytotoxic to syngeneic F4-5 Friend leukemia cells yet were not cytotoxic to DBA/2 lymph node, thymus, and Con A-stimulated spleen cells.

Secondary allogeneic MTLC in both the DBA/2 and C57BL/6 histocompatibility model systems were repeated and the effector cells generated, were retested for their cytotoxic potential against other syngeneic tumor cell targets. Results of these repetitive trials are displayed graphically in Figs. 2 and 3 and in tabular form in Table II. Syngeneic tumor cell lysis was not dependent upon target cell production of infectious MuLV, or upon cell surface expression of RNA tumor virus structural proteins. In the C57BL/6 model system, cytotoxicity was demonstrated against both the low titer virus-producing tumor cell, RBL-5, and the nonproducer FBL-3(Hn) (whose membranes expressed gp-71), as well as against a nonproducer whose cell surfaces lacked detectable concentrations of viral antigens (EL-4). DBA/2 tumor targets were also lysed equally well by syngeneic

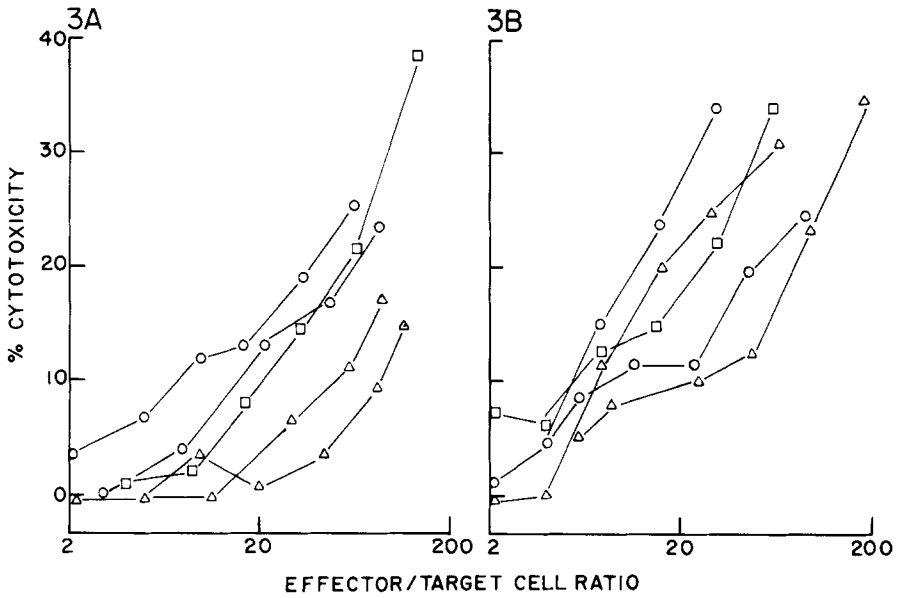

FIG. 3. (A) Lysis of other syngeneic tumor cells effected by C57BL/6 spleen cells after secondary allogeneic MTLC activation. Cytotoxicity is directed against the FLV-induced nonproducer leukemia cells FBL-3(Hn) (□—□), the RLV-induced virus-producing leukemia cell RBL-5 (○—○), and the chemically-induced (Gross virus negative) EL-4 (△—△). (B) DBA/2 lymphocytes selected by secondary allogeneic MTLC and assayed for their cytotoxic potential against syngeneic FLV-induced nonproducer F4-5 cells (□—□), FLV-induced high titer virus producing FLD-3 cells (○—○), and chemically-induced P815 cells (△—△).

effector cells generated in secondary allogeneic MTLC regardless of whether or not they produced MuLV or expressed viral structural proteins on their cell surfaces. All effector cells which demonstrated syngeneic tumor-specific cytotoxicity were sensitive to lysis with anti-theta serum ([mouse AKR-anti-C3H ascites] [no. 8301, Bionetics Laboratory Products, Kensington, Md.]) and absorbed rabbit complement.

Secondary Stimulation with Con A. Further experimentation was designed to discover why secondary allogeneic MTLC stimulation of normal spleen cells was successful in generating syngeneic tumor-specific cytotoxic lymphocytes, whereas secondary MLC and primary allogeneic MTLC were not. It seemed possible that secondary allogeneic MTLC activation might act as a nonspecific restimulator of a small population of cytotoxic cells to proliferate to the point where they were capable of demonstrating cytotoxicity against syngeneic tumor cell targets. To test this hypothesis experiments were conducted in which normal spleen cells, subjected to a single allogeneic MTLC, were harvested and restimulated with Con A (2.5 μg/ml), for an additional 2 days, after which they were tested for cytotoxicity against both syngeneic and allogeneic tumor cells. In addition, viable cells harvested from primary MLC stimulation were restimulated with Con A and tested for their cytotoxic specificities. Other effector cell populations included viable cells harvested from secondary allogeneic MTLC stimulation, and normal spleen cells stimulated with Con A (2.5 μg/ml) for 48 h before their use in cytotoxicity assays. Results of these experiments are dis-

TABLE III

Secondary Stimulation with Con A Lysis of Tumor Cell Targets

Source of spleen cells	Primary stimulation	Secondary stimulation	FLD-3	F4-5	P815	FBL-3(Hn)	RBL-5	EL-4
C57BL/6	F4-5(A-MTLC)*	F4-5(A-MTLC)	93% (25,000)	97% (25,000)	94% (14,705)	29% (295.4)	34% (313.2)	16% (90.7)
C57BL/6	F4-5(A-MTLC)	CON A‡	81% (12,000)	49% (1,470)	71% (3,571)	0% (0.0)	0% (0.0)	0% (0.0)
C57BL/6	CON A	–	0% (0.0)	0% (0.0)	–	0% (0.0)	–	–
C57BL/6	DBA/2ˣ§(A-MLC)	CON A	9% (3.1)	0% (0.0)	18% (5.6)	0% (0.0)	0% (0.0)	0% (0.0)
C57BL/6	DBA/2ˣ(A-MLC)	DBA/2ˣ(A-MLC)	79% (25,000)	44% (862.0)	94% (11,363)	5% (7.1)	0% (0.0)	7% (11.1)
DBA/2	FBL-3(A-MTLC)	FBL-3(A-MTLC)	29% (285.11)	36% (329.2)	28% (275.1)	92% (25,000)	75% (25,000)	32% (263.1)
DBA/2	FBL-3(A-MTLC)	CON A	9% (4.0)	12% (20.8)	8% (25.0)	77% (20,833)	68% (8,333)	20% (62.5)
DBA/2	CON A	–	0% (0.0)	0% (0.0)	–	0% (0.0)	–	–
DBA/2	C57BL/6ˣ(A-MLC)	CON A	0% (0.0)	0% (0.0)	0% (0.0)	25% (166.7)	23% (154.2)	0% (0.0)
DBA/2	C57BL/6ˣ(A-MLC)	C57BL/6ˣ(A-MLC)	5% (7.1)	0% (0.0)	7% (11.7)	86% (25,000)	56% (12,000)	20% (83.3)

* A, allogeneic.

‡ CON A refers to secondary stimulation with the mitogen Con A at a concentration of 2.5 μg/ml.

§ DBA/2ˣ or C57BL/6 refers to irradiated populations of respective spleen cells. Cytotoxicity is expressed in terms of lysis at an effector to target cell ratio of 100:1 and in LU per culture, e.g., 75% (5432.7).

played in Table III. Only cells harvested from secondary allogeneic MTLC stimulation were capable of effecting syngeneic tumor-specific cytotoxicity. Cells harvested from primary allogeneic MTLC and secondary Con A stimulation lysed only allogeneic targets. Spleen cells harvested from primary allogeneic MLC activation and restimulated nonspecifically with Con A were not cytotoxic to syngeneic tumor cells. Normal spleen cells stimulated solely with Con A demonstrated neither syngeneic nor allogeneic tumor cell cytotoxicity. According to these results (Table III), it appeared that the stimulus present in secondary allogeneic MTLC conditions which allowed for generation of tumor-specific cytotoxic lymphocytes was a specific-antigen stimulation which could not be mimicked by mitogen-induced proliferation.

Primary and Secondary Allogeneic MTLC Stimulation of Spleen Cells from Mice Immunized with Allogeneic Nonproducer Friend Leukemia Cells. We questioned whether spleen cells from mice immunized with mitomycin-treated allogeneic Friend leukemia cells and activated in vitro via primary or secondary allogeneic MTLC would be as effective in demonstrating syngeneic tumor-specific cytotoxicity, as were normal spleen cells subjected to secondary allogeneic MTLC. 5- to 7-wk-old normal C57BL/6 female mice were immunized weekly with a subcutaneous inoculum of 5×10^6 mitomycin-treated allogeneic nonproducer Friend leukemia cells (F4-5). Mice were immunized six times. Age-matched DBA/2 female mice were immunized by using an identical immunization schedule and route, with 5×10^6 mitomycin-treated FBL-3(Hn) Friend leukemia cells. 2–4 wk after final immunizations, spleen cells were harvested from immunized mice and stimulated in allogeneic MTLC reactions. After MTLC stimulation viable cells were assayed for cytotoxicity directed against allogeneic and syngeneic tumor cell and syngeneic normal cell targets. Results of experiments involving spleen cells taken from C57BL/6 mice immunized with F4-5 are displayed in tabular form (Table IV).

Spleen cells taken from C57BL/6 mice immunized with F4-5 and activated in vitro in a primary allogeneic MTLC were effective in killing syngeneic tumor cells. Tumor targets lysed included the same FBL-3(Hn), RBL-5, and EL-4 cells used as targets in earlier LMC assays (Fig. 3 A, Table III). The amount of

TABLE IV
Secondary Stimulation of Immune Spleen Cells: Lysis of Tumor Cell Targets

Source of spleen cells	Primary stimulation	Secondary stimulation	FLD-3	F4-5	P815	FBL-3(Hn)	RBL-5	EL-4
C57BL/6	F4-5(A-MTLC)*	F4-5(A-MTLC)	96% (25,000)	92% (25,000)	89% (15,750)	31% (255.1)	37% (454.5)	18% (81.2)
C57BL/6 immunized with F4-5	F4-5(A-MTLC)	–	95% (25,000)	85% (25,000)	90% (12,195)	33% (265.9)	24% (175.2)	17% (113.4)
C57BL/6 immunized with F4-5	F4-5(A-MTLC)	F4-5(A-MTLC)	90% (25,000)	98% (25,000)	87% (11,905)	39% (367.64)	29% (226.5)	15% (92.2)
C57BL/6 immunized with F4-5	–	–	0% (0.0)	0% (0.0)	0% (0.0)	0% (0.0)	0% (0.0)	0% (0.0)

Cytotoxicity is expressed in terms of lysis at an effector to target cell ratio of 100:1 and in LU per culture, e.g., 75% (5432.7).
* A, allogeneic.

syngeneic tumor cell lysis observed was comparable to that observed after secondary allogeneic stimulation of normal spleen cells. Therefore regardless of whether the first allogeneic stimulation was given in vitro or in vivo, secondary allogeneic activation was shown to be capable of producing effector cells which displayed syngeneic tumor-specific cytotoxicity.

Because of this observation, we questioned whether further allogeneic stimulation would produce lymphocytes which expressed greater levels of syngeneic tumor-specific cytotoxicity. Experiments were conducted which subjected lymphocytes, harvested from mice immunized with allogeneic nonproducer leukemia cells, to secondary allogeneic MTLC stimulation (essentially tertiary allogeneic stimulation). Results of these experiments are shown in Table IV. The data collected showed that tertiary allogeneic MTLC stimulation of F4-5 immune cells did not create a population of cells which demonstrated greater syngeneic tumor-specific cytotoxicity than that already present after a secondary allogeneic MTLC. Furthermore, cytotoxicity was not greater than that effected by normal C57BL/6 spleen cells subjected to secondary allogeneic MTLC. Similar results were obtained with lymphocytes harvested from DBA/2 mice immunized with allogeneic FBL-3(Hn) cells and activated in vitro in primary and secondary MTLC. All effector cells which demonstrated syngeneic tumor-specific lysis were sensitive to lysis with anti-theta serum and complement.

Discussion

Results of experimentation conducted in this study may be explained by the following hypothesis: successive MTLC stimulation of a normal lymphoid population leads to the selective proliferation of any number of subpopulations of antigen-specific lymphocytes. Because the antigen stimulation in these repetitive MTLC came from allogeneic nonproducer leukemia cells (which expressed the cell surface viral structural glycoprotein gp-71), a subpopulation of cytotoxic lymphocytes that one would expect to proliferate, would be cells with specificities directed against differing histocompatibility antigens. It is possible that different lymphoid subpopulations would be activated by other antigens present on the surface of the stimulating tumor cells. Our evidence indicates these other antigens are TASA which may be distinct from viral antigens. Because TASA(s)

are presumably shared by all tumor targets, (including total nonproducers), it may be these TASA-reactive lymphocytes which are responsible for producing the syngeneic tumor-specific cytotoxicity observed in LMC assays. We believe that the antigen recognized is a TASA rather than a viral structural protein because the syngeneic tumor target cells lysed by spleen cells harvested from secondary allogeneic MTLC, include chemically, as well as virus-induced tumor cells and also include tumor cells which do not actively product infectious MuLV or express detectable cell surface concentrations of viral structural antigens (gp-71, p-30, or p-12).

Objections might still be raised to claiming that the antigen-reactivity of these tumor-specific cytotoxic lymphocytes is directed against a TASA not necessarily of viral origin. For one, this study's virologic characterization of tumor cell surface viral antigen expression was conducted by using antisera directed against purified RLV antigens, when several of the tumor targets tested were FLV-induced. However, this study (Table I) and others have demonstrated the type-specific cross-reactivity of antisera directed against structural antigens purified from either RLV or FLV (21, 22). It may also seem possible that antigen-reactivity might be directed against a viral structural protein, not assayed for cell surface expression (i.e., p-10 or p-15). Recent studies have shown that p-10 and p-12 are totally cross-reactive as determined by quantitative competitive radioimmunoassay (23). Hunsmann et al. have further demonstrated that p-15 is not expressed at the cell surface of several murine leukemia cells and that the only viral antigens present on the cell surface are the structural glycoprotein gp-71 and the structural proteins, p-30 and p-12 (24). Similar observations have recently been noted in studies conducted in our laboratory involving 15 murine leukemia cell lines maintained in continuous culture (Smith, K. A., M. M. Ferm, and A. E. Gillis, unpublished observations).

It might also seem possible that the TASA responsible for the reactivity of the tumor-specific cytotoxic lymphocyte produced in secondary allogeneic MTLC was associated with either an endogenous or a xenotropic RNA tumor virus produced in all tumor targets. Although we have not directly tested for this possibility, the lack of RT activity (Table I) in culture medium, sampled from nonproducer tumor cell cultures lends evidence that this is not the case.

Regardless of the antigen-specific reactivity of the syngeneic tumor-specific cytotoxic lymphocytes produced in these studies, one must consider possible explanations as to why they are produced only via successive allogeneic MTLC stimulation. A simplistic explanation would be that only after two stimulations have tumor-specific cytotoxic lymphocytes proliferated to the extent where they are detectable in LMC assays. Other experiments conducted in this study point out the problem with this hypothesis. If secondary MTLC stimulation were solely a nonspecific proliferative stimulus, one would expect secondary stimulation (of cells harvested from primary allogeneic MTLC) with Con A to have generated effector cells capable of lysing syngeneic tumor targets. However, this was not the case (Table III). Furthermore, because the antigen(s) recognized by these cytotoxic lymphocytes are presumably shared by syngeneic murine leukemia cells as well, one would expect secondary syngeneic MTLC stimulation to have produced lymphocytes capable of killing syngeneic tumor cells. However,

experimentation (Table II) showed that secondary syngeneic MTLC activation of normal spleen cells was incapable of producing tumor-specific cytotoxic lymphocytes.

An appealing explanation is that the recognition of differing H-2 antigens (in secondary allogeneic MTLC) and the proliferation of anti-H-2 reactive lymphocytes, produces a factor or a culture condition which stimulates the proliferation of other antigen-specific cytotoxic lymphocytes. In other words, repeated allogeneic MTLC stimulation produces a cell-mediated allogeneic effect which may specifically aid in the generation of syngeneic tumor-specific cytotoxic lymphocytes. The allogeneic effect, wherein induction of a graft versus host reaction in an antigen primed animal, greatly enhances production of specific antibody after secondary antigen stimulation, has been associated with the generation of a soluble allogeneic effect factor (25, 26). Dutton et al. showed that supernates harvested from short-term in vitro cultures of histoincompatible mouse spleen cells contained a nonantigen-specific, biologically active mediator, capable of markedly enhacing in vitro antibody responses to thymus-dependent antigens (27). The effects of similarly produced factors have been incorporated into theories regarding T- and B-cell cooperation (28, 29). Other soluble factors produced by incubation of T cells with specific antigens (30) (other than those associated with the major histocompatibility complex) or with nonspecific mitogens (31), have been successful in mediating T- and B-cell collaboration.

Recently, a cell-mediated allogeneic effect has been documented in vitro in studies dealing with immune reactivity to human leukemia (32). With a three cell mix approach, lymphocytes harvested from a patient in remission from acute myelogenous leukemia were cocultured with autologous (previously harvested) irradiated leukemia cells and irradiated allogeneic peripheral blood lymphocytes. Effector cells harvested from three cell mix cultures contained lymphocytes specifically cytotoxic to the patient's autologous leukemia cells. Effector cells harvested from cultures containing only remission lymphocytes and irradiated autologous leukemia cells were incapable of demonstrating autologous leukemia cell cytotoxicity. It was suggested that the success of the three cell mix in generation of leukemia-specific cytotoxic lymphocytes was due to a strong proliferation-inducing stimulus (in this case from the allogeneic irradiated lymphocytes) which when produced, stimulated other cytotoxic lymphocytes. These findings are compatible with the concept of an allogeneic effect.

A similar effect may be operating in the production of syngeneic tumor-specific cytotoxic lymphocytes via secondary allogeneic MTLC stimulation. The allogeneic nonproducer leukemia cell (used as the MTLC sensitizing agent) is at once the source for the production of allogeneic effect stimulation (in that it carries differing H-2 antigens for induction of a large anti-H-2 proliferative response) and the source for presentation of tumor-associated antigens. These are the antigens recognized by the lymphocyte subpopulation which benefits from the allogeneic effect, and later expresses its reactivity against syngeneic tumor cells also bearing TASA(s).

Such an allogeneic effect would explain why other culture treatments were incapable of generating tumor-specific cytotoxic lymphocytes. Secondary syngeneic MTLC stimulation clearly would have been incapable of producing any proliferation-inducing allogeneic effect in that responders and stimulators

shared histocompatibility antigens. Any MLC stimulation would be unsuccessful because the normal lymphocytes used as stimulators lacked TASA(s). Primary allogeneic MTLC either alone or followed by secondary Con A stimulation may not generate enough of an allogeneic effect to successfully promote proliferation of TASA-reactive lymphocytes.

Finally, the procedure of secondary allogeneic MTLC may provide a means of detecting human leukemia associated antigens as well as defining whether cross-reactive human leukemia antigens exist. These are important questions since active immunotherapy with irradiated allogeneic leukemia cells is currently under clinical investigation. Taylor et al. have reported negative studies in acute myelogenous leukemia where attempts were made to generate lymphocytes cytotoxic to autologous leukemia cells after a single allogeneic MTLC (33). Based upon results detailed in this report, it might be expected that a secondary MTLC stimulation would improve the sensitivity of these assays.

Summary

In vivo or in vitro immunity to murine leukemia virus (MuLV)-induced leukemia cells which do not effectively produce virus, has been difficult to demonstrate. Because immunizations with allogeneic murine leukemia cells have been used to confer syngeneic tumor immunity to virus-producing cells, we attempted to generate lymphocytes, cytotoxic to syngeneic nonproducer leukemia cells, by stimulating normal murine spleen cells with allogeneic nonproducer leukemia cells in mixed tumor lymphocyte culture (MTLC) reactions in vitro. Secondary allogeneic MTLC of normal C57BL/6 or DBA/2 spleen cells effectively produced syngeneic tumor-specific cytotoxic lymphocytes. Target cells lysed in lymphocyte-mediated cytolysis (LMC) assays, included both Friend and Rauscher virus-induced syngeneic murine leukemia cells and chemically-induced hematopoietic tumor cells. Syngeneic tumor cells were lysed regardless of whether they produced infectious MuLV or expressed viral antigens gp-71, p-30, or p-12 at the cell surface. Syngeneic normal cells (thymus, lymph node, or Concanavalin A-stimulated spleen cells) used as targets in LMC assays were uneffected by lymphocytes harvested from secondary allogeneic MTLC. Several other in vitro culture treatments including secondary syngeneic MTLC and repetitive mixed lymphocyte culture stimulations were incapable of generating tumor-specific cytotoxic lymphocytes.

Based upon these results, we propose that secondary MTLC stimulation of normal spleen cells with allogeneic nonproducer leukemia cells selects for the proliferation of two subpopulations of antigen-specific cytotoxic lymphocytes. The population capable of effecting syngeneic tumor cell lysis is directed against tumor-associated cell surface antigens which may be distinct from viral structural proteins or glycoproteins. The growth of these tumor-specific cytotoxic lymphocytes may be enhanced by a soluble allogeneic effect factor produced by the proliferation of the second subpopulation of lymphocytes generated in repetitive allogeneic MTLC, namely those lymphocytes with specificities directed against differing histocompatibility antigens.

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