IN VITRO CYTOTOXICITY BY A NONTHYMUS-PROCESSED LYMPHOCYTE POPULATION WITH SPECIFICITY FOR A VIRALLY DETERMINED TUMOR CELL SURFACE ANTIGEN*

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A lymphocyte population may be subdivided into developmental and functional categories. The bone marrow-derived (B) lymphocytes have a high concentration of surface immunoglobulin (Ig)[1] (1–3) and provide the precursors of antibody-secreting cells (4). The thymus-processed (T) lymphocytes, on the other hand, have a very low, if at all detectable surface Ig concentration (1–3) and have the capability to become cytotoxic to target cells bearing histocompatibility antigens against which the T cells have been sensitized (5, 6). It seems likely, however, that cells other than T cells can be killer cells for target cells. For example, in one system using antibody-coated target cells the cell-mediated lysis appears to be a non-T cell function (7, 8).

With the development of methods for isolation of B- or T cell-deficient populations it has been possible to delineate the functions of these cells and some of their interrelationships. Cytotoxicity studies using immune B- and T cell-deficient populations have been performed within the *H-2* system (5, 6) demonstrating T cells to be the dominating killer cells in these tests. With tumor-specific and/or virally determined antigens in syngeneic hosts, T vs. B or non-T cytotoxicity in vitro have not been defined.

Using Moloney sarcoma virus (MSV)-induced tumors in BALB/c mice it has been demonstrated that, from animals in which tumors have regressed, lymphocytes are cytotoxic against cells carrying the Moloney leukemia virus (MLV)-determined surface antigen (9). In the present investigation lymphocyte cytotoxicity was examined against target cells possessing the MLV surface

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[1] *Abbreviations used in this paper:* B cell, bone marrow-derived lymphocytes; FCS, fetal calf serum; Ig, immunoglobulin; MLV, Moloney leukemia virus; MSV, Moloney sarcoma virus; SRBC, sheep red blood cells; T cells, thymus-processed lymphocytes.

antigen, using T- or B cell-deficient subpopulations as well as unfractionated lymphocytes from BALB/c regressor mice.

Materials and Methods

Animals.—BALB/c mice of both sexes 6 wk to 3 months of age were used as a source of lymphocytes. Control animals were matched with immune animals by sex and age.

Virus.—MSV-M (SVRP 224, prepared from the 224th passage in weanling BALB/c mice) was kindly provided by Dr. J. B. Moloney, National Cancer Institute, Bethesda, Md.

Inoculations.—Animals received 0.1 ml of the MSV suspension intramuscularly. Tumors developed between 5 and 10 days and usually regressed by day 25 with a peak tumor size of 10–15 mm about day 15. The lymphocytes were harvested from animals 30 days after MSV infection, 8–10 days after tumor regression. One group of control animals received 0.1 ml of sheep red blood cells (SRBC) intraperitoneally 30 days before testing.

Medium.—Minimal essential medium (Eagle's) with 10% heat-inactivated fetal calf serum (FCS), 100 μg/ml penicillin, and 50 μg/ml streptomycin was used.

Lymphocytes.—Lymph nodes from the axillary and inguinal regions and spleens dissected out under sterile conditions were teased apart in ice-cold medium without FCS, mixed with a Pasteur pipette, and passed over a fine nylon mesh filter. The cells were pelleted at 200 *g* for 10 min and resuspended in tris(hydroxymethyl)aminomethane-buffered 0.75% ammonium chloride, pH 7.2, and incubated for 10 min at 37°C to lyse erythrocytes (10). The cells were washed three times with medium without FCS, resuspended in complete medium, and counted in a hemacytometer using the trypan blue dye exclusion technique to assess the viability.

Preparation of Nonthymus-Processed Lymphocytes.—By using antisera with specificity for mouse T cells it is possible to lyse these cells in the presence of complement, leaving viable cells which are predominantly B cells (11). An anti-T serum produced in a rabbit against mouse thymus cells and absorbed repeatedly with mouse myeloma cells and with repopulated spleen cells from thymectomized, lethally irradiated, bone marrow-protected animals has been described previously (6, 11). Spleen cells remaining after treatment with this antiserum are enhanced in their capability to respond to a thymus-independent antigen, but their ability to respond to a thymus-dependent antigen is decreased by more than 90% (6, 11). This antiserum in the presence of complement will completely abolish the capability of T cells to be cytotoxic in anti-*H-2* tests (6).

In the present experiments, 1–2 $\times$ 10^7 immune or control lymphocytes were incubated with 1 ml of a 1:100 dilution of the anti-T serum for 30 min at 4°C, after which normal rabbit serum at a final concentration of 1:20 was added as a source of complement and incubated for 30 min at 37°C. The anti-T serum has an activity against thymus-derived cells at dilutions as high as 1:10,000 but no activity against bone marrow-repopulated spleen cells at dilutions higher than 1:10 (6, 11). After determination of viability by trypan blue dye exclusion, the cells were pelleted by centrifugation at 600 *g* and suspended in 1.5 ml of 0.25% trypsin. After incubation for 10 min at 37°C, the trypsin was neutralized by addition of medium with FCS and the cell suspension was passed over a nylon mesh filter to remove aggregates of debris forming from the trypsinized dead cells. The cells were then washed twice with medium before use. This procedure removes almost all dead cells leaving a 95–100% viable non-T cell population. The trypsin treatment and washing should also remove any possible anti-T antibodies attached to the remaining cells.

In one experiment the cell population thus prepared was passed through a 5 cm cotton wool column prepared in a Pasteur pipette prewashed with medium at room temperature. In parallel, control lymphocytes from animals which had been immunized against SRBC were also passed through a cotton wool column of the same size and conditions. This procedure should reduce the number of macrophages (12) and mature antibody-secreting cells

(13). 5×10^5 of these cells immune to SRBC were tested for 7S hemolytic plaque-forming cells (14) in duplicate plates before and after passage over cotton wool.

Preparation of T Cells.—By filtering the lymphocytes through glass bead columns, coated first with mouse Ig and then with rabbit anti-mouse Ig in excess, it is possible to trap cells with a high concentration of membrane-bound Ig, i.e., B cells. Thus, the eluted cell population will be primarily T cells (11). Lymphocyte populations passed over such a column have been demonstrated to be predominantly T cells by multiple criteria (6, 11). In the *H-2* system, immune cells passed across such columns have an increased cytotoxicity against the appropriate target cells (6). In the present experiments, an aliquot of the cells eluted from the column were treated with anti-T serum and complement as described above. 85–95% of this subpopulation of cells were killed by this treatment as compared with approximately 50% cytotoxicity of the unfractionated mixture of spleen and lymph node cells. Samples of 2–5 $\times 10^7$ unfractionated lymphocytes from control or immune animals were fractionated by the use of these anti-Ig-coated columns as previously described (6, 11). The number of cells eluted from the columns was 20–30% of the original number. After elution the cells were pelleted by centrifugation, resuspended in complete medium, and counted in a hemacytometer.

Target Cells.—The following cell lines were used: (*a*) Ha2 cells, a line established from an MSV-induced tumor of a CBA mouse. These cells produce both MSV and MLV and possess the MLV-determined surface antigen (15). (*b*) D56 cells, a mixture of S^+L^- (sarcoma-positive, leukemia-negative) cells and normal 3T3 cells from NIH Swiss embryo fibroblast cultures (provided through the courtesy of Dr. R. H. Bassin, NIH, Bethesda, Md.) S^+L^- cells were isolated by appropriate selection procedures from MSV-transformed 3T3 cells (16). The cells contain the MSV genome but do not produce infectious virus nor do they possess a viral-associated surface antigen which would distinguish them from normal 3T3 cells (9). These cells are not killed by MSV immune lymphocytes (9). (*c*) D56-M cells, MLV superinfected D56 cells. The latter two lines are identical except for infectious virus production and the presence of the MLV-associated surface antigen in the D56-M line, which has also been shown to be killed by MSV immune lymphocytes (9).

Determination of Lymphocyte Cytotoxicity.—The microcytotoxicity method of Takasugi and Klein (17) was used. 50–100 target cells were seeded into each well of microplates (No. 3034; Falcon Plastics, Div. B-D Laboratories Inc., Los Angeles, Calif.) and incubated overnight at 37°C in 5% CO_2. The lymphocytes were added to the target cells in ratios of 200:1, 100:1, 50:1, and 25:1. Control and immune lymphocytes were always tested on the same plate using six replicates for each lymphocyte dilution and 12 replicate wells containing no lymphocytes (blank control). After incubation for 48 hr, medium was removed and the plates were washed once with balanced salt solution to remove dead cells. The plates were fixed with methanol and stained with eosin methylene blue and Giemsa, and the target cells remaining in each well were counted (hereafter reference to well indicates the number of target cells in the well). The arithmetic mean and standard deviation of the six replicate wells of each lymphocyte dilution was determined and a Student's t test was performed to assess the significance of the differences between wells containing immune lymphocytes and those containing the same number of control lymphocytes. A significant difference between control and immune wells was considered to exist when the P value was less than 0.05. The relative percentage of remaining target cells in wells containing immune lymphocytes was calculated compared with the wells containing the same number of control lymphocytes. Control and immune lymphocytes and their fractionated subpopulations from individual animals were tested in three separate experiments.

RESULTS

Table I shows the results of the experiments using Ha2 target cells. There was significant killing (P less than 0.05) by the unfractionated lymphocytes in

TABLE I

Activity of Fractionated Lymphocytes from MSV Regressor Mice vs. IIa2 Target Cells Compared with Fractionated Control Lymphocytes

Lympho-cytes	Exp. No.	Blank	Lymphocyte:target cell ratio												Cytotoxic rating
			200:1			100:1			50:1			25:1			
			C	I	%	C	I	%	C	I	%	C	I	%	
Unfrac-tionated	1	137 ± 58	49 ± 15	58 ± 10	120	74 ± 50	56 ± 25	77	100 ± 32	144 ± 39	144	117 ± 55	162 ± 45	138	−
	2	71 ± 21	49 ± 21	37 ± 11	75	82 ± 27	31 ± 13	38[2]	68 ± 23	52 ± 17	76	75 ± 29	45 ± 10	59[1]	++
	3	60 ± 14	63 ± 25	52 ± 19	83	80 ± 32	41 ± 17	52[1]	68 ± 22	49 ± 18	70[1]	85 ± 28	53 ± 29	62[1]	+++
Non-T cells	1	98 ± 37	100 ± 43	58 ± 21	58[1]	64 ± 20	44 ± 32	69	81 ± 43	68 ± 11	83	82 ± 35	71 ± 38	87	+
	2	87 ± 39	90 ± 51	53 ± 23	59	58 ± 13	32 ± 12	54[2]	98 ± 37	49 ± 13	50[1]	62 ± 27	34 ± 7	56[1]	+++
	3a	37 ± 19	45 ± 13	27 ± 5	64[1]	68 ± 21	23 ± 7	34[2]	62 ± 15	27 ± 12	43[2]	76 ± 20	36 ± 15	48[2]	++++
	3b	36 ± 7	70 ± 20	41 ± 17	59[1]	59 ± 34	27 ± 9	46[1]	76 ± 28	34 ± 13	44[2]	57 ± 8	48 ± 12	83	+++
T cells	2	70 ± 26	110 ± 46	53 ± 12	48[1]	89 ± 26	102 ± 36	115	73 ± 27	57 ± 10	78	87 ± 23	72 ± 22	82	+
	3	20 ± 10	44 ± 20	34 ± 18	77	48 ± 19	37 ± 3	77	45 ± 23	29 ± 12	64	52 ± 15	46 ± 15	89	−

Unfractionated, spleen and lymph node cells; non-T cells, prepared using anti-T serum; T cells, prepared using anti-Ig-coated glass bead column.

Blank = No. of target cells ± standard deviation (SD) in wells containing no lymphocytes; C = No. of target cells ± SD in wells containing control lymphocytes; I = No. of target cells ± SD in wells containing MSV immune lymphocytes; % = relative percentage of surviving target cells (I/C × 100).

Superscript numbers represent P values of $1 < 0.05$, $2 < 0.005$.

Cytotoxic rating: significant killing ($P < 0.05$) in 0 dilutions, −; one dilution, +; two dilutions, ++; three dilutions, +++; four dilutions, ++++.

Control lymphocytes in experiments 1 and 2 from unimmunized animals; in experiment 3 control lymphocytes from animals immunized with SRBC; 3a before passage over cotton wool column; 3b after passage over cotton wool column.

TABLE II

Activity of Non-T Lymphocytes from MSV Regressor Mice vs. D56 and D56-MCell Lines

Target cells	Exp. No.	Lymphocyte:target cell cell ratio												Cytotoxic rating
		200:1			100:1			50:1			25:1			
		C	I	%	C	I	%	C	I	%	C	I	%	
D56	2	65 ± 25	71 ± 22	109	46 ± 12	63 ± 45	137	56 ± 11	66 ± 29	128	56 ± 21	49 ± 15	87	−
D56-M	2	118 ± 32	97 ± 19	82	147 ± 43	109 ± 25	74[1]	112 ± 43	71 ± 11	63[1]	95 ± 16	115 ± 40	121	++

D56, MLV antigen-negative cell line; D56-M, MLV antigen-positive cell line; C, I, %, superscript numbers and cytotoxic rating as in Table I.

experiments 2 and 3. In all three experiments the killing was increased by rendering the population deficient of T cells. The relatively pure T cell subpopulations, however, eluted from the columns in experiments 2 and 3 were less cytotoxic to the target cells than the comparable unfractionated lymphocytes. The 200:1 lymphocyte to target cell ratio in experiment 2 showed significant cytotoxicity by the immune T cells, but this was not found at lower ratios.

Donors of control cells in experiment 3 were immunized against SRBC. These lymphocytes produced no killing of the target cells compared with the blank control. In the same experiment MSV immune lymphocytes produced significant killing using unfractionated or non-T cell populations, whereas the immune T cells had no significant cytotoxic activity at any ratio.

The non-T cell killing was demonstrated to be specific for the MLV-associated cell surface antigen by testing against D56 (MLV-negative) and D56-M (MLV-positive) target cells. Table II shows the effects of the non-T cell population from experiment 2 on these lines. There is significant killing by the immune cells of the D56-M line, whereas the immune cells have no cytotoxic effect on the D56 line.

Thus, this killing is specific for the MLV-determined cell surface antigen by the following criteria: (*a*) the MSV immune non-T lymphocytes significantly reduce the number of surviving target cells (Ha2 and D56-M) compared with control non-T lymphocytes from unimmunized animals, (*b*) the immune lymphocytes kill only those target cells which possess the MLV-associated surface antigen (Ha2 and D56-M) but not cells which are MLV antigen free (D56), (*c*) immunization with a nonpertinent antigen (SRBC) produces no killing of the Ha2 target cells.

In order to decrease the number of macrophages and mature antibody-secreting cells, the MSV immune and SRBC immune T cell-deficient preparations were passed over identical cotton wool columns. An aliquot of the SRBC immune cells was tested for SRBC hemolytic plaque-forming cells before and after passage. This procedure decreased the number of plaque-forming cells per 5×10^5 cells by one-third. There was no significant difference in the ability to kill the Ha2 target cells by the MSV immune non-T cells before or after the cotton wool column (Table I, experiments 3a and 3b, respectively) as determined by a Student's *t* test between the wells containing immune lymphocytes on each plate.

DISCUSSION

Lymphocytes from BALB/c mice 30 days after MSV infection and 8–10 days after tumor regression are cytotoxic in vitro against target cells bearing the MLV-determined surface antigen. Fractionation of these lymphocytes at this time shows the most active cell to be a nonthymus-processed cell. The killer cell in this system could conceivably be a non-B cell, however, there is some evidence in favor of a B cell as at least one type of killer cell in this sys-

tem. First, killing cells were excluded from the lymphocyte population by passage over an anti-Ig-coated glass bead column, suggesting a high Ig surface concentration on the active cell population, however, Fc binding receptors on cells other than B lymphocytes (18–20) could also be involved. Secondly, no significant decrease in killing was observed after passage of this subpopulation over a cotton wool column, which should remove or decrease the percentage of macrophages (12).

The mechanism of killing by this non-T subpopulation of cells is unknown. If B lymphocytes are indeed responsible for this killing, membrane-bound Ig on the cell of production in such a system could activate the cell to lyse the target cell by unknown mechanisms after the antibody allowed the killer-target contact (21). On the other hand, if antibody is secreted by the immune cells, this could bind to the target cells and bring about cytotoxicity through the receptor for antigen-antibody complexes on normal B cells (11, 20, 22, 23) and macrophages (19). Such a phenomenon has been demonstrated to exist in several systems (7, 8, 24, 25) including the MSV rat system where target cells pretreated with high concentrations of antisera were lysed by the lymph node cells (26).

Preliminary evidence from time-course studies in this laboratory indicate that T cells also function as killer cells in the present system, but primarily at times closer to tumor development and regression. The present results show the predominant subpopulation of active cells 30 days after MSV infection to be a non-T cell in three separate experiments.

In summary, it appears in this system that tumor cell destruction by lymphoid cells may to a significant extent be mediated by a nonthymus-processed subpopulation (possibly B cells). This would seem to be in contrast to the cell-mediated lysis in the transplantation systems involving major histocompatibility antigens where T cells are reported to be the most significant mediators of target cell destruction (5, 6).

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