THE LYMPHOCYTE RESPONSE TO PRIMARY MOLONEY SARCOMA VIRUS TUMORS IN BALB/c MICE

DEFINITION OF THE ACTIVE SUBPOPULATIONS AT DIFFERENT TIMES AFTER INFECTION*

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(Received for publication 28 February 1973)

Lymphoid cells may be fractionated according to various properties. Macrophages may be removed by their adhesive properties or by allowing them to ingest iron powder and removing them by magnetism (1, 2). The remaining population of lymphocytes consists of the bone marrow-derived, bursa equivalent-processed B lymphocytes and the thymus-processed T lymphocytes. The B cells have a high concentration of surface immunoglobulin (Ig), which is available for detection by anti-Ig reagents (3–5), and provide the precursors of antibody-secreting cells (6). The T cells, on the other hand, have little if any surface Ig that is available for detection by anti-Ig reagents (3–5). T cells can be distinguished antigenically by the presence of the theta (4) or more general thymus-specific antigens (7). Thus, fractionations of lymphocytes into B or T cell-deficient subpopulations may be accomplished by utilizing their surface antigenic characteristics.

By examining the subpopulations of lymphocytes that are active in cellular immunity, it has been demonstrated that T cells are the predominant cytotoxic effector cells in anti-*H-2* tests in one in vitro system (8, 9). Recently, however, some evidence has been presented for heterogeneity in the cytotoxic effector cells in anti-*H-2* tests (10). The lymphocyte subpopulations that are active effector cells in vitro with specificity for tumor-specific and/or virally determined antigens have only recently begun to be defined (11). BALB/c mice injected with Moloney sarcoma virus (MSV)[1] produce

* These investigations were conducted under Contract no. NIH-NCI-E-69-205 within the Special Virus Cancer Program of the National Cancer Institute, National Institutes of Health, U. S. Public Health Service, The Swedish Cancer Society, the Jane Coffin Childs Memorial Fund for Medical Research, and Anders Otto Swärds Stiftelse.

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[1] *Abbreviations used in this paper:* ATG, rabbit antithymocyte globulin; ATS, rabbit anti-mouse thymus serum; FCS, fetal calf serum; MEM, Eagle's minimal essential medium; MLV, Moloney leukemia virus; MSV, Moloney sarcoma virus; NRG, normal rabbit globulin; NRS, normal rabbit serum; S^+L^-cells, sarcoma positive, leukemia negative; SRBC, sheep red blood cells.

lymphocytes that are active against Moloney leukemia virus (MLV) antigen-bearing target cells (12) when the lymphoid cells are taken from animals that have developed no palpable tumors and from animals in which tumors have regressed (13). Lymphocytes from tumor-bearing animals, on the other hand, have been found to be much less active against specific tumor target cells in this system as well as others (13–15). By using lymphocytes from animals 30 days after MSV infection in which primary tumors had regressed 8–10 days before testing, we demonstrated that effector cells that were active in reducing target cell numbers were excluded from the immune cell population by passage over anti-Ig-coated glass bead columns (11). Furthermore, the target cell-reducing effects were increased by rendering the population deficient of T cells using anti-T serum in the presence of complement (11). The activity of these lymphocytes was specific for the MLV-determined tumor cell surface antigen (11–13).

The purpose of the present investigation was to examine in vitro the subpopulations of lymphocytes active in specific tumor target cell reduction through the entire course of development and regression of primary MSV tumors in BALB/c mice and to further characterize the non-T cell involved in this specific anti-tumor cell activity.

Materials and Methods

Animals.—Adult BALB/c mice of both sexes were used as a source of immune and control lymphocytes. Control animals were matched with those that were MSV infected by sex and age in each experiment. 22 MSV-infected and 21 control animals were used in these experiments.

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 in the thigh, after which local tumors developed as early as 5 days, reaching peak tumor size of 10–15 mm about day 15, and usually completely regressing by days 20–25. Lymphocytes were harvested from MSV-infected animals every 5 days for 30 days. In one series of experiments the control animals received 0.1 ml of 0.25% sheep red blood cells (SRBC) intraperitoneally at the time the matched experimental group received MSV. These animals were tested at days 5 and 30.

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

Lymphocytes.—Axillary and inguinal lymph nodes and spleens, dissected out under sterile conditions, were pressed through fine nylon mesh into ice-cold MEM without FCS, mixed with a pasteur pipette, and passed over a fine nylon mesh filter. The cells were pelleted by centrifugation 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 (16). The cells were washed three times in MEM without FCS, resuspended in complete MEM, and counted in a hemacytometer.

Removal of Macrophages (1, 2).—In exp. no. 94 the non-T cell preparation was tested before and after the removal of macrophages. Thereafter, (exps. with numbers higher than 94) macrophages were removed before further fractionation was carried out. After the first wash, the lymphoid cell suspension, 2–4 × 10^8 cells in 5 ml of MEM, was mixed with carbonyl iron and incubated for 30 min at 37°C with gentle agitation every 5 min. The iron was then extracted with the aid of a magnet by passing the suspension through a series of five sterile tubes, each time using the magnetic field to retain the iron in the discarded tube. The cells were then washed twice with MEM before further fractionation. A similar modification of

this technique has been shown to decrease the percentage of macrophages in mouse peritoneal cells from 76 to 86% down to 1% or less.[2] Analysis of the cells at this point by immunofluorescence using rabbit antimouse and goat antirabbit Ig reagents sequentially showed 25–35% of these cells to be surface Ig bearing.

Preparation of Non-T Cells.—Two methods were used to render the lymphocytes deficient of T cells. The first method, using anti-T serum in the presence of complement, has been described previously (11). The second method used an anti-T globulin (ATG) bound to a glass bead column to trap T cells on the column.

Anti-T Serum in the Presence of Complement.—By using the antisera with specificity for mouse T cells it is possible to lyse these cells in the presence of complement leaving viable cells that are predominantly B cells (7). An anti-T serum produced in a rabbit against mouse thymus cells and absorbed repeatedly with mouse myeloma cells and with spleen cells from thymectomized, lethally irradiated, bone marrow-protected animals has been described and characterized previously (7, 9). In the present experiments, $1–2 \times 10^7$ immune or control cells were incubated in a total volume of 0.5 ml with a 1:200 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 mean cytotoxicity from all 20 experiments for unfractionated spleen and lymph node cells by this antiserum was 48.0 ± 14.3% for the immune cells and 44.7 ± 9.1% for the control cells. After determination of the viability by trypan blue exclusion, the cells were pelleted by centrifugation and resuspended in 0.25% trypsin in 0.01 M phosphate-buffered saline, pH 7.4, without calcium or magnesium. After incubation for 10 min at 37°C, the trypsin was neutralized by the addition of MEM with FCS, and the suspension was passed over a fine nylon mesh filter to remove aggregates of debris forming from the trypsinized dead cells. The cells were then washed twice with MEM before use. This procedure removes almost all dead cells leaving a 95–100% viable non-T cell population. Appropriate dilutions of these cells were used for cytotoxicity tests. A sample of these cells was incubated overnight at 37°C in 5% CO_2 in MEM without target cells to allow recovery of surface Ig from the trypsin treatment. These were then analyzed in some experiments for the number of cells bearing surface Ig by immunofluorescence and found to be 85–95% surface Ig bearing.

Preparation of Non-T Lymphocytes Using ATG Columns.—Glass beads with an average diameter of 200 μm (Superbrite 100–5005; Minnesota Mining and Manufacturing Co., St. Paul, Minn.) were cleaned by treatment with equal parts of sulfuric acid and nitric acid; then they were washed and boiled in distilled water. Sterile, filtered ammonium sulfate-precipitated globulin from normal rabbit serum (NRS) was then added at a concentration of 1–2 mg/ml of bead material, suspended in sterile saline. The beads were incubated overnight at +4°C and then poured into 1.5 × 30 cm columns with an approximate volume of 50 ml. A sterile, filtered sheep antirabbit Ig serum (Statens Bakteriologiska Laboratorium, Stockholm, Sweden) was then added to the columns at a dilution of 1:2. The columns were allowed to stand for 1 h at room temperature, were washed off with sterile saline, and filtered with a 1:2 dilution of a rabbit antimouse thymus serum (ATS, cytotoxic titer 1:500,000). After another 1 h at room temperature the columns were washed with sterile saline and ready for use. Control columns were prepared in an identical manner to the ATG columns, except that in the last step NRS 1:2 was substituted for ATS 1:2. Although the ATS, prepared by repeated injections of mouse thymus cells into rabbits over a period of 8 mo, had not been adsorbed with B lymphocytes, the ATG columns prepared with it showed a marked capacity to remove T lymphocytes from mouse lymphocyte populations as shown by morphological and functional tests in Table I. In the present experiments $2–5 \times 10^7$ immune or control lymphocytes were

[2] Golstein, P., and H. Blomgren. 1973. Cells mediating specific in vitro cytotoxicity. III. Further evidence for T cell autonomy. Cytotoxicity of very small amounts of educated thymus cells deprived of macrophages and other non-T cells. *Cell. Immunol.* In press.

TABLE I

Functional and Morphological Characteristics of Mouse Lymphocytes Fractionated by ATG or Anti-Ig Columns

Lymphocytes passed through column with*	GVH function‡		Helper function	
	B cells§	Depression of allogeneic bone marrow	B cells	Antihapten response‖
	%	%	%	
Unfractionated	31	56	—	—
NRG	19	64	38	18.5
NMG	—	—	33	10.4
ATG	82	24	90	1.6
Anti-Ig	<1	56	<1	12.5
No cells	—	—	—	0.5

NRG, normal rabbit globulin; NMG, normal mouse globulin; ATG, rabbit anti-T cell globulin.

* Lymphocytes: lymph node cells from normal CBA mice for GVH (graft-vs.-host) function, or from (CBA × C57BL)F_1 mice immune to bovine serum albumin (BSA) for helper function.

‡ GVH function: 5×10^5 CBA lymphocytes were injected into (CBA × C57BL)F_1 mice that were previously irradiated with 700 R and given 2×10^6 syngeneic bone marrow cells. The ^{59}Fe uptake in spleens was determined 7 days later and expressed in relation to control animals receiving bone marrow cells only.

§ % B cells: percentage of cells with membrane fluorescence using a conjugated goat-antimouse Ig serum.

‖ Antihapten response: antigen-binding capacity in nanograms per milliliter of serum in syngeneic mice given 400 R and injected with 5×10^6 fractionated cells together with 2×10^7 anti-NIP-OA cells and 10 μg of NIP-BSA. Each value is the mean from five to six animals determined 7 days after the cell transfer.

placed on these ATG columns. The cells that passed through (about 30–40% recovery) were used for cytotoxicity tests. Analysis of these preparations by immunofluorescence showed the passed cells to be 85–95% surface Ig bearing.

Preparation of T Cells.—By filtering lymphocytes through glass bead columns, coated first with mouse Ig and then with rabbit antimouse Ig in excess, it is possible to trap cells with a high concentration of membrane-bound Ig, i.e., B cells. Thus, the passed populations will be primarily T cells (7). Lymphocytes eluted from such columns have been demonstrated to be predominantly T cells by multiple criteria (7, 9). In the *H-2* system, immune cells eluted from such a column have an increased cytotoxicity against the appropriate target cells (9). In two other systems, including the present one using regressor mice (11) and a system using chicken erythrocytes coated with soluble antigens as targets (17, 18), specific effector cells were retained by passage through these columns. $2–5 \times 10^7$ lymphocytes from immune or control animals were fractionated by use of these anti-Ig columns as previously described (7). After passage the cells were centrifuged, resuspended in MEM, and appropriate dilutions were used for cytotoxicity tests. In some experiments samples were saved for analysis by immunofluorescence and anti-T serum sensitivity. The mean cytotoxicity of anti-T serum in the presence of complement (at the same concentrations described in the previous section) was 81.3 ± 11.2% for the immune cells and 81.1 ± 9.6% for the control cells. The number of cells bearing surface Ig as detectable by immunofluorescence was reduced to 2–5%.

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 infectious virus, possess the MLV-determined cell surface antigen (19), and have been shown to be sensitive to MSV-immune lymphocytes in microcytotoxicity tests (11–13) but not to lymphocytes immune to a nonpertinent antigen (11, 13). (*b*) D56 cells: a mixture of S^+L^- (sarcoma positive, leukemia negative) cells and normal 3T3 cells from NIH Swiss embryo fibroblast cultures. The cells contain the MSV genome but do not produce any infectious virus nor do they possess a viral-associated surface antigen that would distinguish them from normal 3T3 cells (12), and they are not sensitive to MSV-immune lymphocytes (11–13). (*c*) D56-M cells: MLV-superinfected D56 cells. These cells produce infectious virus, possess the MLV-determined cell surface antigen, and are sensitive to MSV-immune lymphocytes in microcytotoxicity tests (11–13). (*d*) D245-B-3-M: this line, now superinfected with MLV, was originally designated D245-B-3 an S^+L^- line that was isolated by appropriate selection procedures from MSV-transformed BALB/c 3T3 cells (20). The superinfected line produces infectious virus and possesses the MLV-determined cell surface antigen.[3] (Cell lines D56 and D245-B-3 were provided through the courtesy of Dr. R. H. Bassin, National Institutes of Health, Bethesda, Md.)

Determination of Lymphocyte Cytotoxicity.—The microcytotoxicity method of Takasugi and Klein (21) was used with the following modifications: 50–400 target cells were seeded into each well of microplates (no. 3034; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and incubated overnight at 37°C in 5% CO_2. Lymphocytes were added to the target cells in concentrations of 10,000, 5,000, 2,500 and 1,250 per well in 10 μl of MEM. In some experiments the highest concentration was 5,000 and the lowest 625. Control and immune lymphocytes of each fraction were always tested on the same plate using six replicates for each lymphocyte dilution and 6 or 12 replicate wells containing no lymphocytes (blank control). After incubation for 40–48 h, medium was removed and the plates were washed once with balanced salt solution to remove dead cells. The plates were fixed and stained, and the target cells remaining in each well or a representative portion thereof were counted (hereafter reference to well indicates the number of target cells remaining in the well). The log mean and standard deviation (SD) of the six replicate wells 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 wells containing the same number of control lymphocytes using the geometric mean of the six replicate wells for each lymphocyte dilution. Control lymphocytes from 21 animals and immune lymphocytes from 22 animals and their fractionated subpopulations were tested in 20 separate experiments on days 5, 10, 15, 20, 25, and 30 after MSV infection.

EVALUATION OF RESULTS

Because the microcytotoxicity test encompasses several parameters including cytotoxicity, inhibition of target cell growth, target cell detachment without killing, and even stimulation of target cell growth, the evaluation of the results requires special attention.

We have chosen geometric calculations rather than arithmetic to compare the number of target cells surviving in wells with immune lymphocytes and wells with the same number of control lymphocytes, because the test is clearly influenced by exponential cell growth. By counting the number of cells in the

[3] E. M. Fenyö, personal communication.

blank controls using two different target cell concentrations that were stopped at 16 and 48 h or 24 and 48 h, we calculated the doubling time of the Ha2 target cell. Using the mean target cell number from 12–18 samples for each value, we found the mean doubling time to be 10.3 h by the formula:

$g = t(1n2)/1n(C_2/C_1)$, where g = doubling time, t = time interval ($t_2 - t_1$), C_1 = cell number at t_1, C_2 = cell number at t_2.

We selected the absolute number of lymphocytes per well as the parameter for comparison rather than the lymphocyte to target cell ratio for two reasons: First, since the target cells are growing during the test, the ratio does not remain constant. Second, in pilot experiments testing from 40,000 to 312 lymphocytes on 50–200 target cells, we found that no specific effects of the immune cells could be distinguished when using more than 10,000 lymphocytes per well (10^6/ml in a vol of 10 μl). The effects of the normal lymphocytes at these higher concentrations were as great or greater than the immune lymphocytes. This was true regardless of the target cell number. Thus, for these experiments we chose titrations of lymphocytes from 10,000 down to 625 lymphocytes per well.

For unknown reasons, clear titration effects were not seen in most instances using graded lymphocyte numbers. We thus evaluated our results on the basis of statistical significance between immune and control wells rather than using the relative percentage of remaining target cells. In order to be able to compare the activity of an animal's lymphocytes in a single figure, a cytotoxic rating is shown, based on significant target cell reduction ($P < 0.05$) found in zero dilutions $-$, in one dilution $+$, two dilutions $++$, three dilutions $+++$, and four dilutions $++++$. This cytotoxic rating is based on the significant reduction of the target cell numbers as compared with wells with the same number of control lymphocytes. Occasionally there was significant stimulation of cell growth by the immune lymphocytes. These instances are indicated in the results.

Finally, in some cases (4 out of 21 experiments with the unfractionated cells) the normal control lymphocytes were significantly cytotoxic to the target cells when compared with the blank control. However, when comparing the percentage of surviving target cells in wells with control lymphocytes and the blank controls from all experiments, the mean survival of the target cells was 106% of the number of cells in the blank. This value was the same for the unfractionated and both T and non-T fractions. In view of this, one might consider comparing the effects of the immune cells to the blank control only. This approach has some merit since the "normal" lymphocytes in a small number of cases might not be normal because of room infection of these normal animals with C-type viruses. However, the reduction of the number of target cells by the immune lymphocytes compared with normal control lymphocytes is kept as one of our main criteria for specificity. We feel that the need for rigorous specificity controls far outweighs the risk of having a few negative results because of cytotoxicity from the control lymphocytes (in fact, the overall pattern was not

changed by these instances of cytotoxicity from the control lymphocytes). Furthermore, there are a number of unknown factors involved, which may differ from test to test, such as promotion of target cell growth by medium conditioning or by cell-to-cell contact from the added lymphocytes, as well as inhibition of target cell growth by competition for nutrients and accumulation of metabolic wastes by the added lymphocytes. Thus, to ensure that conditions in immune and control wells are as nearly alike as possible including these unknown factors, the only valid comparison is with wells that have the same number of target cells and the same number of immune or control effector cells.

RESULTS

Activity of the Unfractionated Lymphocytes vs. MLV Antigen-Bearing Target Cells.—Table II shows the activity of the unfractionated lymphocytes vs. the three MLV antigen-bearing target cell lines. Experiments up to and including no. 94 were performed using spleen and lymph node cells from which the macrophages had not been removed. Experiments with numbers higher than 94 were performed using lymphocytes that had been rendered macrophage-deficient using carbonyl iron and magnetism. As previously reported in this system (13), the unfractionated lymphocytes from MSV-infected animals were found to be active in specific target cell reduction from animals that had not yet developed tumors at the time of testing and more predictably so, from animals in which tumors had completely regressed. Tumor-bearing animals, in general, were found to have the least active lymphocytes; two exceptions to this are seen in exps. 49 and 104 at 10 and 20 days after MSV infection, respectively. The animals tested at 15 days at peak tumor size, however, were found invariably to have the least active lymphocytes. In exps. 110 and 111 the unfractionated cells were passed over control columns coated with normal rabbit globulin (NRG). Active cells were not excluded by this procedure.

T Cell Activity.—Table III shows the activity of the cells passed through anti-Ig columns vs. the MLV-positive cell lines. These cells show two distinct peaks of activity: just before tumor development on day 10 and just after regression on days 20 and 25. The T lymphocytes from the tumor-bearing animals on days 5 and 20 were found to have a greater activity than the unfractionated cells on the same days. At peak tumor size, however, the T cells were also inactive. By 30 days after MSV infection, passage of the immune cell populations over the anti-Ig columns significantly reduced the inhibitory effects on the target cells in each experiment. In exp. 56 only the highest concentration of T cells was found to produce significant reduction of the Ha2 target cells. This was not found at lower concentrations. In the other two experiments at day 30, significant inhibitory effects were completely eliminated by passing the cells over anti-Ig columns.

Non-T Cell Activity.—Table IV shows the activity of the non-T cells vs. MLV antigen-positive target cells. In exps. 108, 109, and 110 the non-T cells were

prepared by both methods (*a*) by anti-T serum in the presence of complement and (*b*) by ATG columns. All the other non-T cell preparations were fractionated by method *a* only, except exp. 111 in which the cells were fractionated by method *b* only. In exps. 108, 109, and 110 the cells prepared by both methods were found to have identical activities against the target cells. In exps. 108 and 109 both preparations were inactive. In exp. 110 both preparations were found to significantly reduce the target cell numbers in the same three lymphocyte concentrations and the percentage of surviving target cells at each dilution was almost identical.

In exp. 94 the non-T cells were tested before and after the removal of macrophages with carbonyl iron (Table IV 94a and 94b, respectively). Removal of the macrophages clearly increased the inhibitory effects of the non-T cells. In experiments after no. 94, macrophages were removed from the initial unfractionated cells before further fractionation.

Activity is seen in this non-T cell subpopulation both before and after (but not during) peak tumor size; however, at 30 days the non-T cell fraction continued to be active. In three of the four experiments at this time the activity was increased by rendering the lymphocyte population deficient of T cells and in no case at day 30 was there any decrease in the activity by this treatment.

Specificity.—This lymphocyte-mediated target cell reduction has previously been shown to be specific for the MLV-determined cell surface antigen, by the unfractionated lymphocytes throughout the entire 30 day period (13) and by the non-T cells from regressor animals 30 days after virus infection (11). Table V shows the effects of the T cell and non-T cell fractions at day 25 (exp. 55) on the D56 cell line. No significant reduction is demonstrable by either fraction against this cell line that is S^+L^-. Whereas, both these fractions in the same experiment were significantly cytotoxic to the D56-M cell line (Tables III and IV). These two lines are identical except for the production of infectious virus and the presence of the MLV-determined surface antigen on the latter.

In one series of experiments the donors of control lymphocytes were immunized against SRBC in order to determine whether there was a differential activity by the MSV-immune lymphocytes compared with lymphocytes immune to a nonpertinent antigen. The unfractionated MSV-immune lymphocytes on day 30 (exp. 68, Table II), the T cells on day 5 after MSV (exp. 64, Table III), and the non-T cells from an MSV regressor animal on day 30 (exp. 68, Table IV) produced significant reductions of the number of target cells compared with the effects of SRBC-immune lymphocytes that were prepared in the same way. Thus, this lymphocyte-mediated reduction of target cells by either unfractionated, T cells, or non-T cells is specific by the following criteria: (*a*) The MSV-immune lymphocytes significantly reduce the number of surviving target cells (Ha2, D56, D56-M, and D245-B-3-M) compared with control lymphocytes from uninoculated animals. (*b*) The immune lymphocytes are active against only those target cells that possess the MLV-determined surface antigen but not

TABLE II

Activity of Unfractionated Lymphocytes vs. MLV Antigen-Positive Target Cells

Time after MSV	Exp. no.*	Target cell‡	Lymphocyte concentration per well												Cytotoxic rating**	Tumor status‡‡
			10^4			5×10^3			2.5×10^3			1.25×10^3				
			C§	I‖	%¶	C	I	%	C	I	%	C	I	%		
days																
5	97	Ha2	1.483 ±0.201	1.454 ±0.118	93	1.870 ±0.341	1.270 ±0.101	25[5]§§	1.660 ±0.114	1.417 ±0.124	57[4]	1.638 ±0.166	1.486 ±0.236	71	++	0
	92	Ha2	1.851 ±0.115	1.584 ±0.151	54[4]	2.018 ±0.108	1.761 ±0.124	55[5]	1.981 ±0.111	1.915 ±0.112	86	2.109 ±0.192	1.888 ±0.287	60	++	0
	48	Ha2	2.019 ±0.211	1.878 ±0.246	71	2.017 ±0.269	1.868 ±0.229	71	2.130 ±0.396	1.989 ±0.174	72	2.056 ±0.303	2.035 ±0.118	95	−	0
	64	Ha2	0.989 ±0.276	1.101 ±0.118	126	1.205 ±0.212	1.115 ±0.068	81	1.276 ±0.196	1.212 ±0.219	86	1.390 ±0.227	1.275 ±0.302	76	−	T
10	108	Ha2	1.987 ±0.139	2.005 ±0.092	106	2.015 ±0.190	1.888 ±0.307	75	1.777 ±0.181	1.848 ±0.105	118	1.885 ±0.180	1.838 ±0.154	90	−	0
	49	D56-M	1.857 ±0.165	1.618 ±0.149	58[2]	1.887 ±0.140	1.733 ±0.155	70	1.751 ±0.201	1.805 ±0.083	113	1.944 ±0.167	1.683 ±0.167	55[2]	++	T
	94	Ha2	1.704 ±0.194	1.643 ±0.087	87	1.780 ±0.197	1.682 ±0.135	80	1.807 ±0.353	1.951 ±0.205	139	1.913 ±0.138	2.144 ±0.186	170‖‖	−	0
15	109	Ha2	1.874 ±0.127	1.847 ±0.149	94	1.872 ±0.135	1.855 ±0.105	96	1.893 ±0.207	2.012 ±0.112	132	1.869 ±0.116	1.923 ±0.137	113	−	T
	103	Ha2	1.824 ±0.111	1.899 ±0.065	119	1.771 ±0.149	1.780 ±0.105	102	1.573 ±0.335	1.654 ±0.114	106	1.674 ±0.129	1.792 ±0.113	131	−	T
	51	Ha2	1.913 ±0.164	1.843 ±0.191	85	1.816 ±0.130	1.756 ±0.133	87	1.914 ±0.127	1.856 ±0.225	87	1.884 ±0.242	1.947 ±0.203	116	−	T

20	110	Ha2	2.074	2.142	117	2.183	1.948	58	2.054	1.777	53[1]	2.046	1.685	44[4]	++	R
			±0.275	±0.131		±0.204	±0.272		±0.286	±0.174		±0.213	±0.146			
	104	D245-B-3-M	1.657	1.414	57[4]	1.712	1.707	99	1.836	1.707	74	1.734	1.754	105	+	T
			±0.110	±0.134		±0.171	±0.124		±0.136	±0.148		±0.175	±0.072			
	53	Ha2	1.319	1.193	75	1.537	1.413	75	1.694	1.399	51[2]	1.388	1.712	211‖‖	+	R
			±0.211	±0.256		±0.232	±0.130		±0.153	±0.225		±0.167	±0.195			
	53	D56-M	2.072	1.861	62[1]	2.188	1.930	55[1]	2.193	2.165	94	2.110	2.140	107	++	
			±0.130	±0.249		±0.184	±0.250		±0.150	±0.198		±0.220	±0.265			
25	106	Ha2	2.857	2.839	96	2.889	2.788	77[2]	2.792	2.766	94	2.812	2.742	85	+	R
			±0.072	±0.083		±0.074	±0.094		±0.100	±0.080		±0.126	±0.071			
	106	D245-B-3-M	2.324	2.287	92	2.447	2.183	55[4]	2.369	2.167	63[1]	2.391	2.334	88	++	
			±0.247	±0.087		±0.131	±0.124		±0.137	±0.188		±0.067	±0.207			
	55	D56-M	1.878	1.793	82	1.908	1.902	99	1.991	1.973	96	1.980	1.833	71	−	R
			±0.239	±0.201		±0.205	±0.378		±0.201	±0.131		±0.274	±0.147			
	67	Ha2	1.849	1.840	98	1.830	1.836	101	1.603	1.899	198‖‖	1.744	2.081	217‖‖	−	T
			±0.110	±0.157		±0.430	±0.066		±0.207	±0.170		±0.313	±0.105			
30	56	Ha2	1.654	1.549	78	1.891	1.452	36[5]	1.855	1.694	69	1.839	1.640	63[1]	++	R
			±0.205	±0.140		±0.161	±0.224		±0.283	±0.139		±0.210	±0.093			
	68	Ha2	1.768	1.693	84	1.867	1.577	51[2]	1.821	1.658	69	1.911	1.680	59[1]	++	R
			±0.180	±.0153		±0.196	±0.202		±0.158	±0.176		±0.143	±0.211			
	111	Ha2	1.978	1.847	80[1]	1.819	1.960	138	1.925	1.968	111	1.739	1.787	111	+	R
			±0.105	±0.079		±0.172	±0.151		±0.142	±0.190		±0.175	±0.208			

* Experiments with numbers higher than 94: macrophages have been removed.

‡ Target cells: Ha2, D56-M, D245-B-3-M; all MLV antigen positive.

§ C = log mean ± standard deviation (SD) of target cells in wells with control lymphocytes.

‖ I = log mean ± SD of target cells in wells with MSV-immune lymphocytes.

¶ % = relative percentage of surviving target cells based on the formula: geometric mean of immune wells/geometric mean of control wells × 100.

** Cytotoxic rating = significant reduction of target cells in: no dilutions −; one dilution +; two dilutions ++; three dilutions +++; and four dilutions ++++.

‡‡ Tumor status = 0, no palpable tumor; T, tumor bearing; R, regressor.

§§ Superscript numbers represent P values of 1 < 0.05, 2 < 0.025, 3 < 0.01, 4 < 0.005, 5 < 0.0025, and 6 < 0.0005.

‖‖ Significant ($P<0.05$) stimulation of target cell growth.

TABLE III

Activity of T Cells vs. MLV Antigen-Positive Target Cells*

Time after MSV	Exp. no.	Target cell	Lymphocyte concentration per well: 10 C	10 I	10 %	5 × 10 C	5 × 10 I	5 × 10 %	2.5 × 10 C	2.5 × 10 I	2.5 × 10 %	1.25 × 10^3 C	1.25 × 10^3 I	1.25 × 10^3 %	Cytotoxic rating	Tumor status
days																
5	97	Ha2	1.720 ±0.300	1.664 ±0.113	88	1.752 ±0.085	1.794 ±0.334	110	1.705 ±0.128	1.677 ±0.161	84	1.721 ±0.198	1.596 ±0.224	75	−	0
	92	Ha2	1.740 ±0.201	1.890 ±0.130	141	1.872 ±0.226	1.928 ±0.169	114	1.992 ±0.118	1.836 ±0.079	70[2]	1.979 ±0.262	1.850 ±0.183	74	+	0
	48	Ha2	1.595 ±0.244	1.546 ±0.257	89	1.696 ±0.156	1.689 ±0.160	98	1.771 ±0.225	1.663 ±0.289	78	1.746 ±0.140	1.762 ±0.275	104	−	0
	64	Ha2	1.204 ±0.209	1.213 ±0.120	102	1.304 ±0.175	1.043 ±0.305	55[1]	1.183 ±0.150	1.372 ±0.218	154	1.424 ±0.163	1.378 ±0.222	90	+	T
10	108	Ha2	1.458 ±0.182	1.663 ±0.203	160‖	1.478 ±0.163	1.674 ±0.179	140	1.459 ±0.188	1.487 ±0.206	107	1.437 ±0.134	1.646 ±0.175	161‖	−	0
	49	Ha2	1.931 ±0.150	1.630 ±0.193	50[3]	1.864 ±0.163	1.746 ±0.164	76	2.104 ±0.149	1.893 ±0.223	62[1]	2.113 ±0.182	2.090 ±0.181	95	++	T
	49	D56-M	1.646 ±0.087	1.399 ±0.205	49[4]	1.606 ±0.075	1.477 ±0.736	74	1.626 ±0.095	1.541 ±0.174	67[1]	1.533 ±0.183	1.702 ±0.087	148‖	++	T
	94	Ha2	1.906 ±0.199	1.632 ±0.149	53[2]	1.960 ±0.286	1.900 ±0.130	87	2.255 ±0.166	2.312 ±0.303	114	2.310 ±0.282	2.300 ±0.206	97	+	0
15	109	Ha2	1.931 ±0.087	1.878 ±0.055	89	1.941 ±0.097	1.983 ±0.054	110	1.884 ±0.109	1.941 ±0.140	114	1.995 ±0.065	1.998 ±0.086	101	−	T
	103	Ha2	1.651 ±0.049	1.749 ±0.099	125‖	1.603 ±0.185	1.570 ±0.219	93	1.596 ±0.120	1.510 ±0.079	82	1.575 ±0.169	1.562 ±0.122	97	−	T
	51	Ha2	1.719 ±0.132	1.583 ±0.193	136	1.759 ±0.210	1.960 ±0.159	159	1.772 ±0.191	1.921 ±0.152	141	1.882 ±0.166	1.864 ±0.161	96	−	T

20	110	Ha2	1.821 ±0.283	1.791 ±0.235	93	2.114 ±0.181	1.732 ±0.131	41[5]	1.957 ±0.249	1.729 ±0.268	59	2.029 ±0.198	1.792 ±0.179	58[1]	++	R
	104	D245-B-3-M	1.899 ±0.127	1.694 ±0.061	62[4]	1.874 ±0.099	1.914 ±0.154	110	2.064 ±0.209	1.849 ±0.094	61[2]	2.077 ±0.111	1.881 ±0.136	64	+++	T
	53	D56-M	2.089 ±0.228	1.899 ±0.166	64[2]	2.116 ±0.228	2.021 ±0.110	81	2.008 ±0.183	2.032 ±0.188	106	2.090 ±0.102	1.977 ±0.191	77	+	R
25	106	Ha2	2.651 ±0.067	2.590 ±0.099	87	2.703 ±0.104	2.534 ±0.134	68[2]	2.731 ±0.070	2.598 ±0.134	74[4]	2.577 ±0.080	2.593 ±0.141	104	++	R
	106	D245-B-3-M	2.199 ±0.180	2.150 ±0.169	89	2.240 ±0.235	1.977 ±0.073	55[2]	2.318 ±0.180	2.167 ±0.153	71	2.357 ±0.147	2.112 ±0.114	58[3]	++	R
	55	D56-M	1.896 ±0.148	1.873 ±0.238	95	1.951 ±0.241	1.612 ±0.158	46[3]	1.947 ±0.089	1.859 ±0.170	81	1.884 ±0.073	1.923 ±0.125	109	+	R
	67	Ha2	1.610 ±0.125	1.624 ±0.140	103	1.610 ±0.118	1.787 ±0.116	150[1]	1.632 ±0.233	1.851 ±0.206	166	1.965 ±0.151	1.957 ±0.184	98	−	T
30	56	Ha2	2.005 ±0.196	1.709 ±0.116	51[4]	1.935 ±0.123	1.932 ±0.322	100	1.844 ±0.144	1.691 ±.0275	71	1.949 ±0.257	1.838 ±0.135	78	+	R
	68	Ha2	1.607 ±0.198	1.488 ±0.212	76	1.651 ±0.167	1.502 ±0.235	71	1.601 ±0.220	1.410 ±0.274	64	1.699 ±0.136	1.643 ±0.198	88	−	R
	111	Ha2	2.051 ±0.168	2.022 ±0.105	94	2.056 ±0.099	2.074 ±0.072	104	2.044 ±0.043	2.059 ±0.114	104	2.005 ±0.109	2.047 ±0.122	110	−	R

Target cells, C, I, %, superscript numbers, cytotoxic rating, and tumor status as in Table II.

* T cells prepared using anti-Ig-coated columns. (2–5% surface Ig positive.)

TABLE IV

Activity of Non-T Cells vs. MLV Antigen-Positive Target Cells

Time after MSV	Exp. no.*	Target cell	Lymphocyte concentration per well: 10 C	10 I	10 %	5×10^3 C	5×10^3 I	5×10^3 %	2.5×10^3 C	2.5×10^3 I	2.5×10^3 %	1.25×10^3 C	1.25×10^3 I	1.25×10^3 %	Cytotoxic rating	Tumor status
days																
5	97	Ha2	2.172 ±0.129	2.138 ±0.120	95	2.223 ±0.179	2.185 ±0.273	92	2.295 ±0.374	2.429 ±0.093	136	2.236 ±0.230	2.133 ±0.243	79	−	0
	92	Ha2	2.031 ±0.207	1.830 ±0.210	63	1.937 ±0.177	1.862 ±0.164	84	2.051 ±0.070	1.845 ±0.228	62[1]	1.939 ±0.088	1.935 ±0.083	99	+	0
	48	Ha2	1.597 ±0.173	1.408 ±0.153	65[1]	1.721 ±0.212	1.234 ±0.198	33[5]	1.999 ±0.104	1.527 ±0.252	34[5]	1.904 ±0.259	1.621 ±0.213	52[1]	++++	0
	64	Ha2	1.393 ±0.192	1.488 ±0.134	124	1.446 ±0.261	1.333 ±0.115	77	1.383 ±0.282	1.297 ±0.203	82	1.593 ±0.213	1.514 ±0.266	84	−	T
10	108a	Ha2	1.973 ±0.192	1.911 ±0.214	87	1.608 ±0.297	1.649 ±0.245	110	1.566 ±0.217	1.622 ±0.194	114	1.458 ±0.262	1.482 ±0.214	106	−	0
	108b	Ha2	1.655 ±0.092	1.972 ±0.073	207‡‖	1.629 ±0.185	1.588 ±0.214	91	1.459 ±0.265	1.507 ±0.249	112	1.468 ±0.184	1.483 ±0.108	104	−	0
	49	D56-M	1.958 ±0.124	1.818 ±0.153	72	2.021 ±0.157	1.671 ±0.127	45[5]	1.926 ±0.139	1.732 ±0.108	64[2]	1.980 ±0.172	1.803 ±0.134	67[1]	+++	T
	94a	Ha2	1.538 ±0.237	1.717 ±0.191	151	1.839 ±0.323	1.836 ±0.192	106	1.640 ±0.298	1.631 ±0.723	98	1.719 ±0.294	1.421 ±0.279	50[1]	+	0
	94b	Ha2	1.681 ±0.127	1.411 ±0.065	56[6]	1.965 ±0.184	1.531 ±0.217	37[5]	2.442 ±0.283	1.981 ±0.295	35[2]	2.579 ±0.132	1.887 ±0.223	20[6]	++++	0
15	109a	Ha2	1.970 ±0.201	1.984 ±0.095	103	1.960 ±0.093	1.930 ±0.088	94	1.886 ±0.247	1.933 ±0.173	111	1.913 ±0.163	1.951 ±0.092	109	−	T
	109b	Ha2	1.784 ±0.043	1.866 ±0.059	121‡‖	1.754 ±0.058	1.716 ±0.078	91	1.780 ±0.126	1.754 ±0.140	94	1.731 ±0.157	1.746 ±0.058	104	−	T
	103	Ha2	1.980 ±0.053	2.061 ±0.159	112	1.777 ±0.110	1.895 ±0.198	141	1.607 ±0.270	1.786 ±0.240	131	1.665 ±0.103	1.739 ±0.110	121	−	T
	51	Ha2	1.445 ±0.248	1.633 ±0.173	154	1.473 ±0.251	1.529 ±0.233	114	1.690 ±0.166	1.640 ±0.168	89	1.511 ±0.237	1.602 ±0.159	123	−	T

20	110a	Ha2	2.268 ±0.240	2.245 ±0.178	95	2.274 ±0.193	1.870 ±0.189	40[5]	2.063 ±0.235	1.784 ±0.234	53[1]	2.672 ±0.134	1.713 ±0.250	44[3]	+++	R
	110b	Ha2	1.748 ±0.166	1.738 ±0.319	98	1.983 ±0.097	1.532 ±0.192	35[5]	2.134 ±0.204	1.859 ±0.208	53[2]	2.013 ±0.104	1.489 ±0.185	30[6]	+++	R
	104	D245-B-3-M	1.821 ±0.188	2.047 ±0.198	168‖‖	1.961 ±0.066	1.824 ±0.098	73[3]	2.052 ±0.131	2.064 ±0.168	103	2.043 ±0.123	2.101 ±0.073	114	+	T
	53	D56-M	1.940 ±0.142	1.956 ±0.690	106	2.019 ±0.138	1.857 ±0.208	69	1.923 ±0.173	1.835 ±0.165	82	2.156 ±0.072	1.951 ±0.210	62[2]	+	R
25	106	Ha2	2.928 ±0.082	2.841 ±0.073	82[1]	2.903 ±0.069	2.896 ±0.076	98	2.930 ±0.033	2.874 ±0.178	88	2.890 ±0.078	2.830 ±0.081	87	+	R
	106	D245-B-3-M	2.294 ±0.173	2.181 ±0.264	77	2.280 ±0.060	2.282 ±0.090	100	2.356 ±0.162	2.256 ±0.054	79[1]	2.460 ±0.131	2.224 ±0.125	58[4]	++	R
	55	D56-M	1.963 ±0.208	1.876 ±0.261	82	1.792 ±0.085	1.845 ±0.175	113	1.833 ±0.165	1.861 ±0.056	107	2.117 ±0.266	1.829 ±0.109	52[2]	+	R
	67	Ha2	1.651 ±0.117	1.603 ±0.226	90	1.662 ±0.076	1.739 ±0.156	118	1.745 ±0.139	1.604 ±0.125	72[1]	1.660 ±0.181	1.741 ±0.210	121	+	T
30	39	Ha2	2.034 ±0.260	1.774 ±0.237	55[1]	2.070 ±0.179	1.606 ±0.229	34[5]	1.939 ±0.362	2.134 ±0.238	157	2.036 ±0.332	2.117 ±0.281	121	++	R
	56	Ha2	1.898 ±0.235	1.682 ±0.213	61	1.690 ±0.255	1.474 ±0.157	61	1.961 ±0.174	1.712 ±0.244	56[1]	1.759 ±0.180	1.494 ±0.234	54[1]	++	R
	68	Ha2	1.831 ±0.117	1.574 ±0.211	55[2]	1.724 ±0.212	1.410 ±0.173	48[3]	1.846 ±0.206	1.498 ±0.169	45[4]	1.754 ±0.062	1.667 ±0.096	82[1]	++++	R
	111b	Ha2	1.941 ±0.147	1.719 ±0.205	60[1]	1.946 ±0.092	2.016 ±0.072	118	1.937 ±0.091	1.864 ±0.138	85	1.951 ±0.194	1.856 ±0.148	81	+	R

Target cells, C, I, %, superscript numbers, cytotoxic rating, and tumor status as in Table II.

* Exp. 94a before removal of macrophages with iron powder. Exp. 94b after removal of macrophages with iron powder. Experiments with numbers higher than 94: macrophages were removed before further fractionation. In all experiments with numbers lower than 108 and exps. 108a, 109a, and 110a, the non-T cells were prepared using anti-T serum in the presence of complement. In exps. 108b, 109b, 110b, and 111, the non-T cells were prepared using ATG columns. Non-T cells were 85–95% surface Ig positive.

TABLE V

T or Non-T Cells vs. D56 (S^+L^-) Control Target Cells

Time after MSV	Exp. no.	Target cell*	Lymhocyte concentration per wall: 5 × 10³ C	5 × 10³ I	5 × 10³ %	2.5 × 10³ C	2.5 × 10³ I	2.5 × 10³ %	1.25 × 10³ C	1.25 × 10³ I	1.25 × 10³ %	0.625 × 10³ C	0.625 × 10³ I	0.625 × 10³ %	Cytotoxic rating	Cell fx	Tumor status
days																	
25	55	D56	1.669 ±0.318	1.611 ±0.316	87	1.792 ±0.230	1.662 ±0.198	74	1.362 ±0.277	1.402 ±0.311	110	1.342 ±0.380	1.320 ±0.262	95	–	T cells	R
		D56	1.603 ±0.207	1.866 ±0.144	183‖‖	1.816 ±0.213	1.853 ±0.277	109	1.619 ±0.214	1.728 ±0.292	129	1.524 ±0.238	1.556 ±0.392	108	–	Non-T cells	R

C, I, %, cytotoxic rating, and tumor status as in Table II.
* Target cell D56: MLV antigen negative.

TABLE VI

Time Required In Vitro for Significant Effects to Appear

Time after MSV	Exp. no.	Target cell	Lymphocyte concentration per well: 10⁴ C	10⁴ I	10⁴ %	5 × 10³ C	5 × 10³ I	5 × 10³ %	1.5 × 10³ C	1.5 × 10³ I	1.5 × 10³ %	1.25 × 10³ C	1.25 × 10³ I	1.25 × 10³ %	Cytotoxic rating	Time in vitro	Fraction
days																*h*	
25	106a	Ha2	2.111 ±0.284	2.004 ±0.178	78	1.976 ±0.216	1.824 ±0.159	71	1.932 ±0.229	2.086 ±0.250	133	1.848 ±0.132	1.901 ±0.185	113	–	16	Unfx*
			1.843 ±0.054	1.903 ±0.185	115	1.784 ±0.148	1.927 ±0.136	139	1.853 ±0.076	1.964 ±0.117	129‖‖	1.904 ±0.170	1.873 ±0.183	93	–	16	T‡
			2.186 ±0.060	2.021 ±0.113	68[3]	2.382 ±0.122	1.908 ±0.171	40[6]	2.276 ±0.143	2.025 ±0.216	56[2]	1.970 ±0.211	2.051 ±0.054	120	+++	16	Non-T§
	106b	Ha2	1.810 ±0.183	1.729 ±0.135	83	1.603 ±0.287	1.708 ±0.333	128	1.885 ±0.169	1.690 ±0.211	64	1.843 ±0.315	1.741 ±0.203	79	–	24	Unfx
			2.009 ±0.122	2.028 ±0.109	104	2.014 ±0.088	1.952 ±0.107	87	2.110 ±0.088	1.971 ±0.219	73	1.774 ±0.363	2.060 ±0.123	193‖‖	–	24	T
			2.006 ±0.143	1.894 ±0.152	77	1.871 ±0.165	1.818 ±0.138	89	2.021 ±0.143	1.886 ±0.101	74[1]	1.989 ±0.075	1.801 ±0.127	65[3]	++	24	Non-T
	106c	Ha2	2.857 ±0.072	2.839 ±0.083	96	2.899 ±0.074	2.788 ±0.094	77[2]	2.792 ±0.100	2.766 ±0.080	94	2.812 ±0.126	2.742 ±0.071	85	+	48	Unfx
			2.651 ±0.067	2.590 ±0.099	87	2.703 ±0.104	2.534 ±0.134	68[2]	2.701 ±0.070	2.598 ±0.070	74[4]	2.577 ±0.080	2.593 ±0.141	104	++	48	T
			2.928 ±0.082	2.841 ±0.073	82[1]	2.903 ±0.069	2.896 ±0.076	98	2.930 ±0.033	2.874 ±0.178	88	2.890 ±0.078	2.830 ±0.081	87	+	48	Non-T

C, I, %, and cytotoxic rating as in Table II.
* Unfx: (unfractionated) spleen and lymph node cells deprived of macrophages.
‡ T cells: prepared using anti-Ig columns.
§ B cells: prepared using anti-T serum in the presence of complement.

cells that are MLV antigen free. (*c*) The activity of the MSV-immune lymphocytes is significant compared with the effects of SRBC-immune cells in the same experiments.

The Time Required In Vitro for Significant Effects to Appear.—25 days after MSV infection in a regressor mouse when both T and non-T cell fractions were found to be active, an experiment was performed to determine the time required in vitro using the microcytotoxicity test for each fraction, as well as the unfractionated cells, to show significant effects. The results of this experiment are shown in Table VI. Using the same target cell, we stopped the experiment at 16, 24, and 48 h after adding the lymphocytes. Two distinct patterns emerged. While the T cell fraction and the unfractionated lymphocytes required 48 h for the detection of significant reduction of target cell numbers, the non-T cells produced significant reduction as early as 16 h and the effects were less detectable as the incubation time increased.

DISCUSSION

We have presented an analysis of the immune competence of lymphoid cells from BALB/c mice undergoing a primary outgrowth and regression of Moloney sarcoma virus-induced tumors to selectively inhibit the growth of MLV antigen-bearing target cells in vitro. We have attempted to demonstrate the relative roles of T and non-T lymphocytes in the specific inhibition as affected by time after administration of MSV in vivo.

In order to selectively produce "pure" T lymphocytes, cells were filtered through columns coated with anti-Ig, known to remove the B lymphocytes while leaving the T lymphocytes in the passed population (7). Normally such anti-Ig columns will remove more than 90% of B lymphocytes as judged by morphology and function (7), and correspondingly, according to surface antigens a lymphocyte population consisting of more than 90% T lymphocytes is obtained in the passed population (7). This passed population can be shown in vivo as well as in vitro to have the expected specific immune capacity of normally functioning T lymphocytes (7), and there is no evidence that such cells have been damaged by the passage through the anti-Ig columns. If mouse anti-*H-2*-immune cells are filtered through such columns and subsequently tested for cytolytic activity in vitro against relevant, *H-2*-incompatible target cells, there is normally an increase in the activity of the passed cells in comparison with the original population (9).

Two different approaches were used to purify B lymphocytes from a mixed population of lymphocytes. The first approach was using selective lysis of T cells by anti-T serum plus complement, followed by trypsin treatment (to remove dead cells and antigen-antibody complexes) and recovery at 37°C (11). The other approach took advantage of a column separation technique where triple layers of material provided a high concentration of anti-T cell antibodies in the outermost layer. Such columns can be shown to selectively deplete the

passing cells of T lymphocytes as judged both by morphology and function (present article and [22]). The two methods of preparing T cell-deficient subpopulations produce cells that are 85–95% surface Ig positive, and the MSV-immune non-T cells prepared by either method behave in an identical manner with respect to their target cell-reducing capacities. The possibility had previously existed that immune cells fractionated using anti-T serum in the presence of complement could somehow be activated by the antiserum, complement, and/or trypsin and further washing. This was not very likely since the control lymphocytes were treated in the same way without inducing any activity against the target cells (11). Also, the very same antiserum in the presence of complement completely abolished the ability of T cells to be cytotoxic to target cells in anti-*H-2* tests (9). Furthermore, the MSV-immune lymphocytes prepared in this way were active against MLV antigen-bearing target cells but not against control target cells that were MLV antigen free (11). Finally the identical behavior in microcytotoxicity tests of the cells prepared by ATG columns further validates the conclusion that the specific in vitro activity of these cells is indeed a property of this fraction of immune cells rather than a possible product of in vitro manipulations.

All the lymphocyte fractions studied in this system were least active if taken from tumor-bearing hosts at peak tumor size (day 15). Several possibilities exist that might explain this phenomenon. All the active lymphocytes might be aggregated at the tumor site (23) or specific lymphocyte hyporeactivity could have been induced by antigen overload (24). One possibility, which seems particularly compelling in this system, is that otherwise normal lymph node and spleen cells might in fact be antigenically converted by the virus to possess the MLV-associated surface antigen. It has already been demonstrated that viremic animals, injected with MLV as neonates, have normally functioning lymphocytes that are MLV antigen positive (25). Immunizing such animals with SRBC and treatment of the viremic animals' spleen cells with anti-MLV serum in the presence of complement reduces the number of plaque-forming cells; whereas, the same treatment of the control SRBC-immune spleen cells has no effect (25). Large tumors in the MSV system are known to shed infectious virus (26). If, as a result of viremia, a significant percentage of the lymphocytes are MLV antigen positive in the tumor-bearing host, when lymphocytes are added to the target cells, this number of MLV antigen-positive lymphocytes could conceivably mask any inhibitory effects of the simultaneously present immune lymphocytes. This possibility is currently under investigation in this laboratory.

The T cell activity in this system appeared to be related in time to have a peak just before tumor development and just after regression and in some cases when small tumors were present. In two instances, with animals bearing tumors, the T cell activity was greater than the corresponding unfractionated or non-T cell reactions in the same experiments. In none of these experiments with early or late tumor-bearing animals was there any decrease in specific antitarget cell

activity by passage of the cells over anti-Ig-coated columns. The T cell activity in this system, thus, seems to be transient, fading rather rapidly after regression. The fact that T cells are active in this system is not surprising. T cells have been shown to be active cytotoxic effector cells in the transplantation systems where immunization has taken place across major histocompatibility barriers (8, 9).

Non-T cells, on the other hand, have been reported to be active killer cells in antibody-coated target cell systems (27, 28) where there seems to be a general requirement for the addition of sensitizing antibody to the target cells (27–30). The cytotoxicity can then be affected by normal lymphoid cells (27–30) and appears to be dependant on Fc receptors present on the surface of the non-T cells (31). In recent reports in the present system (11) and a nontumor system using soluble antigens bound to target cells (17, 18), no extrinsic antibody was used; only sensitized lymphocytes were added to the target cells. In both these systems, passage of the immune cells over anti-Ig columns decreased their cytotoxic or inhibitory effects on the target cells indicating a non-T effector cell.

The non-T cells active in this system could conceivably be some cell other than a B cell. However, there is increasing evidence in favor of B cells being active as effector cells in this system. Active effector cells can be excluded at day 30 after virus infection on anti-Ig columns indicating a high concentration of surface Ig on the active cell at this time. Fc receptors on cells other than B cells (32, 33) could be involved in the retention, although, as yet this has not been found with these columns (7). Also, the fact that neither ATG columns nor NRG columns inhibited specific antitarget cell activity would tend to exclude this. The non-T cells prepared by ATG columns or anti-T serum in the presence of complement were 85–95% surface Ig positive. The 5–15% of these preparations with no detectable surface Ig are unlikely to be the active effector cells, since significant target cell reduction has been observed with as few as 1,250 lymphocytes. This would mean that the active effector cell would have to be present in concentrations as low or lower than the target cells, and the number in this population that was committed to this specific antigen would be even lower. We had previously reported that decreasing the percentage of macrophages from the non-T cell fraction by passage over a cotton wool column did not decrease the activity of this subpopulation of immune cells (11). In the present experiments, eliminating macrophages using carbonyl iron and magnetism, in fact, increased the activity against the target cells by the non-T cell fraction of immune cells compared with the control lymphocytes treated the same way.

The time required in vitro for significant effects to appear with each fraction would seem to indicate that the non-T cells in this sytem have a more rapid mechanism of action compared with the T cells. 48 h incubation was required for observation of significant T cell activity in this system, which is compatible with previous investigations using the microcytotoxicity method for anti-*H-2*

tests (22). Antibody secreted by immune B cells in this system might be expected to have a quite rapid effect.

The mechanism of reduction of target cell numbers by these cells is unknown. If B cells are indeed responsible for this specific activity of the immune non-T cells in this system, the binding of effector cell to target cell could take place by two different mechanisms. Antibody could be secreted by a few immune cells that would bind to the target cells and bring about cytotoxicity through the receptor for antigen-antibody complexes on normal B cells (7, 34–36). Such a phenomenon has been demonstrated to exist in several systems (27–31) including the MSV rat system where target cells pretreated with antisera were reduced in numbers by the lymph node cells (37). On the other hand, the specific immune B cells might be able to lyse the target cell directly by unknown mechanisms after the membrane-bound Ig has established the killer to target contact (38).

The cellular immune response to this primary viral tumor as measured in vitro by microcytotoxicity tests against MLV antigen-bearing target cells is a heterogenous one involving both T and non-T (probably B) lymphocytes. The T cell response is most active close to tumor development and regression. The B cell activity also begins early after MSV infection, is depressed at peak tumor size (as is T cell activity), emerges again after regression, and remains high late in the immune response even as the T cell activity tapers off.

The present experiments have raised several questions including whether non-T cell activity is a predominant feature of all tumor systems or is just a peculiarity of the present one. Preliminary evidence from this laboratory indicates that non-T cells are also active in specific in vitro target cell reduction in the methylcholanthrene-induced sarcoma system of mice. Also, evidence using tumor targets and lymphocytes from patients in a similar assay suggests non-T lymphocyte involvement.[4]

Finally, a note of caution should be added. There exists ample evidence that T cells are capable of in vivo cytolytic activity against foreign cells (39, 40). It remains to be established if a similar effect can be demonstrated by specific non-T cells or B cells, although preliminary support for such a mechanism exists (41).

SUMMARY

Adult BALB/c mice were injected with Moloney sarcoma virus (MSV) after which the animals' lymphocytes were examined for activity against Moloney leukemia virus (MLV) antigen-bearing target cells at 5-day intervals for 30 days. Lymphocytes from these animals and appropriately matched controls were fractionated into B cell-deficient (primarily T cells) and T cell-deficient (primarily B cells) subpopulations. Macrophages were removed using iron

[4] O'Toole, C., P. Perlmann, H. Wigzell, and B. Unsgaard. 1973. Lymphocyte cytotoxicity in bladder cancer. No requirement for thymus derived cells. *Lancet.* In press.

powder and magnetism. The unfractionated lymphocytes, T cells, and non-T cells were then tested in microcytotoxicity tests. Antigen-specific activity was found in the unfractionated lymphocytes from animals that had not yet developed palpable tumors and from regressor animals. The T cells were active just before tumor development and just after regression; however, by day 30 after virus infection (8–10 days after regression) the T cell subpopulation was much less active. The non-T cell subpopulation was also active before tumor development and soon after regression. However, this activity continued to rise after regression and was highest at 30 days. At day 15 (peak tumor size) neither subpopulation was active. The activity was demonstrated to be specific for the MLV-determined cell surface antigen by testing on control target cells that were MLV antigen negative and by comparison of the inhibitory effects with lymphocytes immune to a nonpertinent antigen as well as normal lymphocytes. The non-T cells were tested for activity before and after removal of macrophages with iron powder and magnetism. Such cells were significantly more active after removal of the macrophages. These data demonstrate specific T cell and non-T cell activity in microcytotoxicity tests with a tumor-specific system and strongly suggest that the non-T cell activity described herein is a B cell function.

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