NATURALLY OCCURRING LYMPHOCYTE-MEDIATED IMMUNITY TO ENDOGENOUS TYPE-C VIRUS IN THE MOUSE

Blocking of the Lymphocyte Reactivity with Antisera to the Virus

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Genetic sequences coding for type-C RNA viruses exist in a naturally integrated form within the DNA of mouse cells (1). In some inbred mouse strains, endogenous viruses infectious for mouse cells are spontaneously activated and replicate in the animal. In such situations, there is an associated incidence of lymphoreticular neoplasia that is correlated with the age on onset and level of virus expression (2, 3). Further, structural proteins of the endogenous type-C viruses have been found in a high incidence in naturally occurring tumors of all histologic types in two strains of low leukemia incidence mice (4). This ubiquitous expression of viral proteins in tumor cells and the finding that at least two of the viral structural proteins, gp70 and p12, are immunologically reactive on the surface of virus infected cells (5) has suggested the possibility that the host's immune response to its endogenous virus might be of importance in determining its immunity to naturally occurring tumors. The host's naturally occurring cell-mediated and humoral immunity has been examined by several groups of investigators. Natural cell-mediated cytotoxic reactivity of lymphoid cells against syngeneic and allogeneic tumor cells containing type-C viruses has been found to reside in specific lymphocyte subpopulations (6–9). In addition, many normal mice have been shown to possess antibodies directed against cell surface antigens associated with type-C viruses (10–12) and against the viruses or purified viral proteins (13–16).

In the present study, we demonstrate a virus-specific lymphocyte mediated immune response induced in response to endogenous type-C virus expression in aging mice. This response is mediated by a specific population of lymphocytes and coexists with humoral antiviral immune responses in the same host. In the process of attempting to define the interaction of these two immune responses, it was found that preincubation with autologous mouse sera having antiviral reactivity blocks the lymphocyte mediated cytotoxicity. Moreover, highly specific heterologous antisera to type-C viral proteins contained high-titered blocking activity which could be removed by absorption with purified viral proteins, thus establishing the virus specific nature of the target antigens for both the lymphocyte cytotoxicity and the blocking activity.

Materials and Methods

Animals. Inbred normal male or female BALB/cCr mice of various ages were used for

natural immunity studies. The mice were bred and aged at the Microbiological Associates, Walkersville, Md., facility. Weanling BALB/c mice were used to prepare immune lymphocytes by injection i.m. of $\cong 10^5$ focus-forming U/0.1 ml of a Moloney leukemia virus pseudotype of Moloney sarcoma virus (MSV [MuLV]).[1] Regressor mice were sacrificed 10–14 d after injection of the virus and their spleens removed for preparation of attacker cells.

Antiserum. Individual or pooled normal mouse serum was collected from the sacrificed mice, aliquoted, and stored at −70°C. Antiserum to R-MuLV gp70, p30, p15, p12, and p10 were prepared according to published procedures (17, 18). Hyperimmune BALB/c anti-R-MuLV serum was obtained by immunizing 2–3-mo-old BALB/c mice with 12 doses of Tween-80 (Atlas Chemical Industries, Inc., Wilmington, Del.)-ether disrupted R-MuLV. The first injection was administered subcutaneously using 200 μg virus mixed with an equal volume of Freund's Complete Adjuvant. The remaining immunizations, 50 μg virus each, were given by the i.p. route at 2–4-wk intervals.

Target Cells. The WM-7 cell line is a virus-free tumor cell line derived from a spontaneously occurring tumor of the BALB/c mouse (19). Subcultures of WM-7 were productively infected with either an endogenous BALB/c-derived ecotropic virus or with the Rauscher (R) strain of murine leukemia virus (MuLV) and are designated B-WM-7 and R-WM-7, respectively (19). Normal rat kidney (NRK) cells have been described (20). A feline embryo fibroblast line designated FFc60WF was obtained from Naval Biological Research Laboratories, Oakland, Calif. and subcultures were productively infected with FeLV. Cell lines were constantly monitored (at each cytotoxicity assay) for the presence of virus by measuring poly (rA) oligo (dT)-directed reverse transcriptase activity in culture fluids (21). The virus tropism and the percentage of infected cells was determined periodically by an infectious center assay (22, 23).

Virus. Sucrose density gradient-purified R-MuLV was obtained from the Frederick Cancer Research Center, Frederick, Md. through the courtesy of R. V. Gilden. The JLS-V9-grown virus was partially purified with two cycles of zonal centrifugation and concentrated either 1,000-fold (vaccine for anti-R-MuLV preparation) or 5,000-fold (for serum absorption). The whole (1,000-fold concentrated) and lysed virus had complement fixation titers of 1:160 and 1:640, respectively, against anti-R-MuLV-p30 and total protein concentration of 2.1 mg/ml. Formalin-inactivated, Tween-80-ether-disrupted R-MuLV vaccine was prepared as previously described (24).

Virus Infectivity Assay. Lymphocyte suspensions from spleens were inoculated at 5×10^6 cells/plate on 24-h cultures containing DEAE-dextran-treated SC-1 wild mouse embryo cells. Plaques were scored with the XC-assay procedure (21) at the 7th d using duplicate plates. Passage on Day 7 and 14 was performed by scraping cells from one tissue culture plate into 0.6-ml supernatant culture fluid. Fresh duplicate 24 h cultures of SC-1 cells were subsequently inoculated with 0.2 ml of the resulting cell suspension and tested 7 d later by the XC-assay.

Preparation and Pretreatment of Lymphocytes. Spleen and thymus were minced under sterile conditions in RPMI-1640 (National Institutes of Health Media Unit, Bethesda, Md.), passed through cotton gauze, washed twice with RPMI-1640, and resuspended in RPMI-1640 containing 20% fetal bovine serum (FBS) and glutamine. To determine the effect of antiserum and complement on the effector cells, $5–10 \times 10^7$ spleen lymphocytes were incubated with 1:2 final dilution of AKR anti-theta C3H ascetic serum (Bionetics Laboratory Products, Litton, Bionetics Inc., Kensington, Md.) in RPMI-1640 with 10% FBS for 30 min at room temperature. The cells were washed once and the cell pellet was resuspended in a 1:3 dilution of nontoxic rabbit serum as the complement source (Grand Island Biological Co., Grand Island, N. Y.) and incubated for 45 min at 37°C. The cells were washed twice, counted, and adjusted to 10^7 cells/ml concentration for cytotoxicity assay at a ratio of surviving attacker: target cell of 200:1. The phagocytic cells were removed with carbonyl iron according to the method published (25, 26). For the removal of the nylon-adherent cells, the spleen cells were passaged over a nylon column prepared with teased, washed fibers from Fenwal Leukopaks (Fenwal Inc., Walter Kidde & Co. Inc., Ashland, Mass.). The columns were first washed with Hank's balanced salt solution and then with RPMI-1640 medium with 10% FBS. The cells at a concentration of 10^7/ml suspended in RPMI-1640 with 10% FBS were added to the column. After incubation, the column was washed with 10 ml medium, the effluent cells were collected and used as attacker cells. For

[1] *Abbreviations used in this paper:* FBS, fetal bovine serum; MSV, Moloney sarcoma virus; MuLV, murine leukemia virus; NRK, normal rat kidney; R, Rauscher.

removal of cells with receptors for complement or immunoglobulins, spleen cell suspensions were incubated on monolayers containing sheep erythrocytes and anti-E antibodies—Cordis Laboratories, Miami, Fla., plus complement according to the published methods (27, 28). The nonadherent cells were collected from the supernates. The cell concentration was adjusted to 10^7 cells/ml and used for the cytotoxicity assay.

Cellular Cytotoxicity Assay. The microcytotoxicity version of the published procedure was employed (29). Target cells (1.0×10^7 cells/ml) were labeled with 200 μCi of ^{51}Cr (200–500 mCi/mg sodium chromate specific activity, New England Nuclear, Boston, Mass.). 5×10^3 labeled cells/0.1 ml volume suspended in RPMI-1640 with 10% FBS and 1×10^6/0.1 ml spleen or thymus lymphocytes were delivered into Falcon Micro-Test II wells (BioQuest, BBL, & Falcon Products, Bectin, Dickinson, & Co., Cockeysville, Md.). The test was incubated for 18 h. The microplate was then centrifuged at 1,500 rpm for 10 min, and 0.1 ml from each well was sampled into 10 ml of Aquasol-2 (New England Nuclear) and counted for 10 min in a Beckman LS-350 beta counter (Beckman Instruments, Inc., Fullerton, Calif.). Each sample in the assay was performed in quadruplicate and the % specific lysis computed as follows:

$$\% \text{ isotope release} = \frac{\text{cpm of supernate} - \text{cpm counter background}}{\text{total cpm incorporated into cells} - \text{cpm counter background}} \times 100.$$

% specific lysis
= % isotope release of spleen lymphocytes − % isotope release of thymus lymphocytes.

The % specific lysis was considered to be positive when a comparison of the means of the spleen lymphocyte samples with the control thymus lymphocyte samples gave a P value of 0.01 or less by the Dunnett test.

Blocking of Cell-Mediated Cytotoxicity. Serum dilutions in RPMI-1640 in 0.1 ml volumes were added to the target cells and incubated for 60 min at 37°C in the presence of 5% CO_2. After incubation, 10^6/0.1 ml lymphocytes were added and the test was incubated for 18 h. The % specific lysis was calculated as described above. Blocking was evaluated by a comparison of the % specific lysis obtained with spleen lymphocytes with the % specific lysis obtained with spleen lymphocytes in the presence of serum. The blocking was considered to be significant when a comparison of the means gave a P value of 0.01 or less calculated by the Dunnett test. The blocking titer of a serum was defined as the highest dilution of the serum which gave significant blocking as defined above.

Humoral Cytotoxicity Assay. The assay used was a modification of previously published procedures (5, 30). The assay was carried out in Micro-Test II plates using 5×10^3/0.1 ^{51}Cr-labeled target cells, 0.1-ml serum dilutions, and 0.1 ml of 1:4 dilution nontoxic rabbit serum (complement). At the end of the assay the plates were centrifuged for 5 min at 1,500 rpm, placed on crushed ice, and 0.1 ml of each was transferred to counting vials containing 10 ml Aquasol-2 (New England Nuclear, Boston, Mass.). The titer was given by the highest serum dilution in which the percentage specific lysis was significant at the 99% confidence level using the conservative Dunnett test.

Immunoprecipitation of ^{125}I-Labeled Type-C Virus Structural Proteins. The isolation and ^{125}I-labeling of R-MuLV structural proteins, including *gag* gene-coded components, p30, p15, p12, and p10, and the envelope glycoprotein, gp70, has been previously reported (17). Immunoprecipitation assays were performed by testing sera at serial twofold dilutions for ability to bind ^{125}I-labeled viral proteins (10,000 cpm). Reaction mixtures contained 0.01 M Tris-HCl, pH 7.8, 1.0 mM EDTA, 0.4% Triton X-100, 1% bovine serum albumin, and 0.05 M NaCl in a total volume of 0.2 ml. In assays using ^{125}I-labeled p15 and gp70 the concentration of NaCl was increased to 0.2 M. After incubation for 3 h at 37°C and 18 h at 4°C, 0.025 ml pig anti-goat immunoglobulin G was added to each tube and incubation continued for a further 3 h at 37°C and 1 h at 4°. Samples were subsequently diluted to 1 ml with cold 0.01 M Tris-HCl, pH 7.8, 0.1 M NaCl buffer containing 0.1% Triton X-100, antigen-antibody complexes precipitated by centrifugation at 2,500 rpm for 15 min, supernates aspirated, and radioactivity in supernates measured in a Searle model 1285 gamma counter (Searle Diagnostics, Des Plaines, Ill.).

Results

Age-Related, Cell-Mediated Cytotoxicity against Cells Shedding Endogenous Ecotropic

TABLE I

*Age-Related Natural Cytotoxicity of BALB/c Spleen Lymphocytes against Target Cells Shedding Ecotropic Class I or Rauscher-MuLV**

Source of lymphocytes (age of mice)	Pool No.	Percent specific lysis against						
		B-WM-7	R-WM-7	WM-7	R-NRK	NRK	FeLV FF 60WF	FF 60WF
mo							*c*	*c*
1	1	<2.0	<2.0	<2.0	4.5	4.2	4.6	5.4
1	2	<2.0	<2.0	<2.0	2.5	2.9	3.6	3.8
6	1	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
6	2	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
12	1	3.3	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
12	2	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
18	1	19.3†	15.1†	<2.0	13.4†	<2.0	4.2	<2.0
18	2	23.9†	16.1†	<2.0	12.8†	<2.0	3.1	<2.0

* Spleen and thymus pools from five mice each were prepared and suspended in RPMI-1640, 1.0×10^7 cells/ml. 1.0×10^6 attacker lymphocytes and 5.0×10^3 labeled target cells were incubated in Micro Test II wells for 18 h at 37°C. The microplate was then centrifuged at 1,500 rpm for 10 min and 0.1 ml from each well was sampled into 10 ml of Aquasol-2 and counted for 10 min in a Beckman LS-350 beta counter. All samples were tested in quadruplicate.

‡ The percent-specific lysis was calculated as presented in Materials and Methods and was considered to be significant when a comparison of the means of the spleen lymphocyte samples with the control thymus lymphocyte samples gave a P value of ≤ 0.01.

Virus. Spleen lymphocytes were obtained from normal BALB/c mice ranging from 1 to 18 mo old. Lymphocyte pools were prepared from five mice and tested in a chromium release cytotoxicity assay against a variety of syngeneic and allogeneic target cells that were virus-free or infected with murine and feline type-C viruses. Lymphocyte pools from 18-mo-old mice showed a striking virus specific activity against syngeneic target cells (WM-7) productively infected with either an endogenous ecotropic BALB/c virus (B-WM-7) or R-MuLV (R-WM-7) (Table 1). Noninfected syngeneic target cells showed no activity against lymphocytes from this age group or any of the age groups tested. Lymphocytes obtained from 1 mo and 6-mo-old mice showed no activity against B-WM-7 or R-WM-7 whereas those from 12-mo-old mice exhibited low level activity. Allogeneic rat cells, noninfected (NRK) and R-MuLV infected (R-NRK) also gave age-related, virus-specific reactivities when tested against lymphocyte pools from 18-mo-old mice (Table 1). Of interest was an apparent low level allogeneic reactivity exhibited by lymphocytes from only 1-mo-old mice that was not related to the virus. Allogeneic cat cells, noninfected FFc60WF, and FeLV-infected (FeLV-FFc60WF), were also tested against attacker lymphocytes from BALB/c mice of various ages. Only lymphocytes from 18-mo-old mice gave low level, but significant reactivity against FeLV infected but not the noninfected cell lines, and again only lymphocytes from 1-mo-old mice gave a nonspecific allogeneic reactivity.

Attempts to Ablate the Virus-Specific Lymphocyte-Mediated Cytotoxicity. Lymphocyte pools were prepared from spleens of 18-mo-old mice and exposed to a variety of treatments in an attempt to identify the specific lymphocyte population that was mediating the cytotoxic reactivity. Treatment of the spleen lymphocyte suspensions with anti-θ serum and complement had no effect on the cytotoxic activity of the lymphocytes directed against the B-WM-7 target cells (Table II). The activity of the

TABLE II

*Effect of Anti-T-Cell Serum or Removal of Lymphocyte Subpopulations on the Cytotoxic Activity of Lymphocytes Obtained from Normal 18-Mo-Old BALB/c Mice**

Treatment	Source of Lymphocytes					
	18-mo-old BALB/c‡				Immune positive control§	
	Pool 1		Pool 2		Pool 1	
	B-WM-7	WM-7	B-WM-7	WM-7	B-WM-7	WM-7
	percent-specific lysis against					
None	11.4‖	<2.0	10.1‖	<2.0	14.6‖	<2.0
Anti-T and complement	8.9‖	<2.0	8.1‖	<2.0	2.1¶	<2.0
Complement	10.5‖	<2.0	10.4‖	<2.0	12.8‖	<2.0
Nylon column	14.8‖	<2.0	13.7‖	<2.0	13.7‖	<2.0
EA	11.7‖	<2.0	8.4‖	<2.0	11.8‖	<2.0
EA complement	13.1‖	<2.0	9.1‖	<2.0	11.0‖	<2.0
Carbonyl iron	12.1‖	<2.0	10.2‖	<2.0	10.4‖	<2.0

* The cell-mediated cytotoxicity assays were performed as described in Table I.

‡ A spleen lymphocyte pool from 25 BALB/c mice of 18-mo-old was prepared and was divided for the respective treatments which are described in Materials and Methods.

§ Weanling BALB/c mice were given an i.m. inoculation of 10^5 focus-forming units of MSV virus. Spleens were removed from the regressors 10–14 d after injection.

‖ As in Table I.

¶ The percent-specific lysis was considered to be significant when a comparison of the means of the immune spleen lymphocyte samples with the control normal spleen lymphocyte samples gave a P value of ≤ 0.01.

anti-θ serum was confirmed by treatment of immune lymphocytes from BALB/c mice in which an MSV (MuLV)-induced tumor had regressed. The anti-θ sera significantly reduced the cytotoxic activity of these immune lymphocytes as compared to non-treated immune lymphocytes tested against B-WM-7 (Table II). Treatment of the normal or immune lymphocytes with complement alone or with carbonyl iron did not significantly affect the cytotoxic activity nor did passage of the lymphocyte suspensions through a nylon column. For removal of cells with receptors for complement or immunoglobulins, the spleen cell suspensions were also incubated on monolayers containing sheep erythrocytes and EA with and without complement. These treatments did not significantly affect the cytotoxic reactivity of lymphocytes from 18-mo-old mice nor of immune lymphocytes from MSV regressor mice against the B-WM-7 target cells.

Relationship of Natural Virus-Specific, Cell-Mediated and Humoral Immunity to Endogenous Virus Expression in the Host. Individual BALB/c mice ranging in age from 1 to 18 mo were examined for expression of endogenous ecotropic virus and its relationship to their virus-specific, cell-mediated, and humoral immune status. Endogenous ecotropic virus was quantitated by cocultivation of 5×10^6 live spleen lymphocytes with SC-1 cells and scoring for plaques both at 7 d and subsequent to subculturing and incubation for an additional 7 or 14 d. By this sensitive assay the endogenous virus incidence (and titer—data not shown) was found to increase progressively with age with the 1-mo-old mice yielding no isolates of 5 tested and the 18-mo-old mice, 4 isolates of 5 tested (Table III). The lymphocyte mediated cytotoxicity to B-WM-7 increased progressively with age as did the humoral immune response to viral antigen

TABLE III

*Age-Related Natural CMI and Humoral Immunity to Ecotropic Virus by Cytotoxicity and Radioimmunoassays: Relationship to Endogenous Virus Expression**

Age of mice	Animal No.	Ecotropic‡ virus	Precipitating§ antibody	Lymphocyte-mediated cytotoxicity‖	Antibody-mediated cytotoxicity¶	
				Percent-specific lysis against		
			gp70	B-WM-7	B-WM-7	WM-7
mo						
1	1-5	−	<10	<2.0	<2.0	NT
6	1-4	−	<10	<2.0	<2.0	NT
	5	+	20	<2.0	<2.0	NT
12	1	+	20	5.4**	<2.0	NT
	2	−	<10	<2.0	<2.0	NT
	3	+	<10	<.20	<2.0	NT
	4	+	20	7.1**	3.1	NT
	5	−	<10	<2.0	<2.0	NT
18	1	+	40	15.6**	5.1†	<2.0
	2	+	80	19.4**	4.3	<2.0
	3	+	160	7.3**	6.1†	<2.0
	4	−	40	4.2	2.0	<2.0
	5	+	80	10.1**	2.0	<2.0

* Individual serum was assayed for precipitating and cytotoxic antibodies and the autologous spleen lymphocytes for cellular cytotoxicity and for ecotropic virus expression.

‡ 5×10^6 spleen lymphocyte suspensions were inoculated onto 5×10^6 SC-1 wild mouse embryo cells. The plaques were scored by the XC-assay at day 7. Subcultures of SC-1 cells were also passaged at 7 and 14 d and assayed 7 d later by the XC assay.

§ Sera were assayed at serial twofold dilutions for ability to bind ^{125}I-labeled purified gp70 antigen. Results are expressed as the reciprocal of the highest dilution at which 10% of the ^{125}I-labeled antigen was precipitated.

‖ Cellular cytotoxicity assays were performed as described in Table 1.

¶ Humoral cytotoxicity was carried out in Micro-Test II wells using quadruplicate samples of 5×10^3/0.1 ml ^{51}Cr-labeled target cells, 0.1-ml serial dilutions of BALB/c serum and 0.1 ml of nontoxic dilutions of rabbit serum (complement). The percent-specific lysis and its significance was calculated as previously described (5).

** As in Table I.

as measured by precipitating activity to the major viral glycoprotein gp70 and by humoral cytotoxic activity to B-WM-7 cells. A high degree of correlation was found between the presence of infectious virus and the virus-specific immune responses in the individual mice. The data suggested further that the immune activity was in response to endogenous virus expression as the immune activity was measurable only in mice which yielded infectious virus. Viral-specific, cell-mediated, and humoral immune activity occurred in the same mice but no definite quantitative relationship of one activity with the other was observed.

Inhibition of the Cytotoxic Activity of Spleen Lymphocytes with Autologous Sera. To determine whether autologous mouse sera would inhibit the lymphocyte mediated cytotoxicity, target cells, including B-WM-7, R-WM-7, and WM-7 are preincubated with various dilutions of sera for 1 h before measurement of lymphocyte-mediated activity. The cytotoxic activity of lymphocytes from 18-mo-old mice was significantly reduced by preincubation of either B-WM-7 or R-WM-7 with serum pools from 18-mo-old

TABLE IV

Blocking of the Cytotoxic Activity of Spleen Lymphocytes from 18-Mo-Old BALB/c Mice with Autologous or Virus-Immune Sera

Sera*	Lymphocytes from 18-mo-old BALB/c			Lymphocytes from 1-mo-old BALB/c		
	Percent-specific lysis against					
	R-WM-7	B-WM-7	WM-7	R-WM-7	B-WM-7	WM-7
None	17.4	19.6	3.4	<2.0	<2.0	<2.0
1-mo-old B/c	16.4	17.0	<2.0	<2.0	<2.0	<2.0
18-mo-old B/c	9.2‡	8.4‡	<2.0	<2.0	<2.0	<2.0
18-mo-old B/c (absorbed with R-MuLV)	16.2	17.2	<2.0	<2.0	<2.0	<2.0
18-mo-old B/c (absorbed with R-gp70)	15.8	17.9	<2.0	<2.0	<2.0	<2.0
B/c anti-R-MuLV	6.4‡	9.1‡	<2.0	<2.0	<2.0	<2.0
B/c anti-R-MuLV (absorbed with R-MuLV)	15.1	15.3	<2.0	<2.0	<2.0	<2.0
B/c anti-R-MuLV (absorbed with WM-7 cells)	7.1‡	9.5‡	<2.0	<2.0	<2.0	<2.0

* Normal or immune sera were mixed with equal volumes of 10 mM Tris HCl (pH 7.8), containing 1.0 mg of disrupted R-MuLV or 50 μg of R-MuLV gp70 and incubated for 1 h at 37°C and 18 h overnight at 4°C, followed by centrifugation at 100,000 *g* for 2 h. Immune sera were also mixed with an equal volume of cell pack, incubated for 1 h at 37°C and 18 h overnight at 4°C, followed by centrifugation at 1,500 rpm for 10 min. BALB/c anti-R-MuLV immune sera was produced as previously published (43).

‡ The blocking of cellular cytotoxicity was performed by adding quadruplicates of 0.1 ml serial serum dilutions into 5.0×10^3 target cells. After 1-h incubation, 1.0×10^6/0.1 ml lymphocytes were added into the microplate wells containing the target cells and the serum and the test was incubated for 18 h. Blocking was evaluated by a comparison of the percent-specific lysis obtained with spleen lymphocytes with the percent-specific lysis obtained with spleen lymphocytes in the presence of serum dilutions. The blocking was considered to be significant when a comparison of the means gave a P value of ≤ 0.01.

mice. This blocking activity of the serum was completely absorbed with intact R-MuLV or with highly purified R-MuLV-gp70 (Table IV). Sera from 1-mo-old mice had no blocking activity against the cytotoxic lymphocytes obtained from 18-mo-old mice. Lymphocytes from 1-mo-old mice were not cytotoxic to B-WM-7 or R-WM-7 and preincubation of the target cells with sera from young or old mice did not affect this reactivity. Immune sera obtained from BALB/c mice hyperimmunized with inactivated R-MuLV significantly blocked the lymphocyte-mediated cytotoxic reactivity. Moreover, absorption of this immune sera with R-MuLV removed this blocking activity whereas absorption with WM-7 cells did not (Table IV).

Analysis of Blocking Activity of Individual Autologous Mouse Sera; Correlation of Blocking Activity with Humoral Cytotoxic Activity. Individual mouse sera were tested for ability to block their autologous lymphocyte's reactivity to B-WM-7. Examination of 10 individual mice yielded 7 whose sera gave statistically significant ($P < 0.01$) blocking when tested at a dilution of 1:10 or greater (Table V). Of these latter sera, six were found to be reactive in a humoral cytotoxicity assay, whereas the three sera having no blocking activity were all unreactive in the cytotoxicity assay. Further, the sera yielding the highest blocking titers also were generally noted to yield the highest cytotoxicity titers, although titers obtained in the latter assay were never found to exceed 1:40.

Blocking of the Cytotoxic Activity of Spleen Lymphocytes with Antisera Specific for the Polypeptides of R-MuLV. High-titered monospecific antisera directed against individual R-MuLV structural proteins were tested for blocking activity to determine whether virus specific reactivities by the spleen lymphocytes could be blocked by antisera specific for the viral proteins and if so, to determine which polypeptide

TABLE V

Analysis of the Blocking Activity of Individual Autologous Mouse Sera: Correlation of Blocking Activity with Humoral Cytotoxic Activity

Mice No.	Lymphocytes	Lymphocytes with autologous sera	*P* value of blocking	Cytotoxicity titer‡ of autologous sera	Blocking titer§ of autologous sera ($P \leq 0.01$)
	percent-specific lysis*				
	B-WM-7	B-WM-7			
1	17.3	7.7	0.005 (S)	20	160
2	12.7	10.6	0.294 (NS)	<10	<10
3	14.8	4.6	0.006 (S)	20	40
4	13.7	8.6	0.014 (S)	10	10
5	11.2	10.9	0.831 (NS)	<10	<10
6	21.4	5.4	<0.001 (S)	40	640
7	18.1	7.1	0.003 (S)	10	40
8	14.3	4.1	0.005 (S)	20	160
9	15.1	6.2	0.007 (S)	<10	10
10	10.9	10.7	0.922 (NS)	<10	<10

* Spleen lymphocytes from individual animals were assayed for cell-mediated cytotoxicity as described in Table I and for blocking of the cytotoxicity with the autologous serum as described in Table IV. The blocking was considered to be significant when a comparison of the means gave a *P* value of ≤0.01.

‡ The humoral cytotoxicity assay was performed as described in Table III using the respective autologous serum. The titer was given by the highest dilution of the serum in which the percent-specific lysis was significant at $P \leq 0.01$.

§ The blocking titer is expressed as the highest serum dilution at which the percent-specific lysis obtained with spleen lymphocytes was significantly different ($P \leq 0.01$) from the percent-specific lysis obtained with spleen lymphocytes in the presence of the test serum.

specific antisera were active in the blocking assay. Table VI shows the reactivities of each polypeptide specific antisera with highly purified preparations of each of the major structural proteins of R-MuLV as determined by radioimmunoassay. It is seen that high-titered blocking activity was observed in anti-R-gp70 sera and in anti-R-p12 sera (Table VI). Whereas anti-R-p15 and anti-R-p30 sera also gave significant but low level activities, the possibility that this was due to the presence of low levels of contaminating anti-p12 reactivity could not be excluded. Anti-R-p10 was highly monospecific and was nonreactive in the blocking assays. It should be noted, however, that the blocking activity of even the most active sera was not complete even when tested at low dilutions (1:10). For example, at antisera dilutions of 1:10, percent specific lysis values of 6.1 and 6.8 were obtained for anti-R-gp70 and anti-R-p12, respectively, as compared to 18.9 for normal goat sera (Table VI).

Removal of blocking and precipitating activity of anti-R-gp70 and anti-R-p12 by absorption with purified gp70 and p12. To further establish the specificity of blocking activity of sera directed against R-MuLV structural proteins, absorptions of such antisera with purified viral proteins was carried out. The results (Table VII) show that the blocking activities of anti-R-MuLV-gp70 and anti-R-MuLV-p12 were reduced by at least two logs by absorption with their respective homologous proteins. In contrast, absorption with heterologous proteins (i.e., absorption of anti-R-MuLV-gp70 sera with p12 and anti-R-MuLV-p12 with gp70) had no significant effect on the blocking activities. The reduction of the precipitating activities of the antisera by absorption was highly correlated with the reduction of their respective blocking activities.

TABLE VI

*Blocking of Spleen Lymphocyte Cytotoxic Activity by Antisera Directed against Individual R-MuLV Structural Proteins**

Sera	Antibody titer as measured by						
	Immunoprecipitation of ^{125}I-labeled					Blocking reactivity	percent-specific lysis (1:10)
	gp70	p15	p12	p30	p10		
Control	<10	<10	<10	<10	<10	<10	18.9
Anti-R-MuLV:							
gp70	80,000	<10	<10	160	<10	>5,120	6.1‡
p15	<10	5,120	160	1,280	<10	320	9.4‡
p12	<10	<10	1,280	<10	<10	5,120	6.8‡
p30	20	<10	160	750,000	80	80	10.3‡
p10	<10	<10	<10	80	3,500	<10	18.1

* Immunoprecipitation of ^{125}I-labeled viral proteins was performed as described in Methods. Results are expressed as the highest serum dilution at which 10% precipitation of the appropriate ^{125}I-labeled antigen was achieved (1,000 cpm as compared to a background level of 50–150 cpm) and represent mean values of triplicate determinations. Blocking assays were performed as described in Materials and Methods. Results are expressed as the highest serum dilution at which the percent-specific lysis obtained the spleen lymphocytes was significantly different ($P \leq 0.01$) from the percent-specific lysis obtained with spleen lymphocytes in the presence of the test serum.

‡ As in Table III. See footnote **.

TABLE VII

Removal of Blocking Activity in Anti-R-MuLV gp70 and p12 Sera by Absorption with the Homologous Protein

Sera	Absorbed with*	Antibody titer as measured by:‡		
		Immunoprecipitation of ^{125}I-labeled		Blocking reactivity
		p12	gp70	
control	—	<10	<10	<20
anti-R-MuLV gp70	—	<10	80,000	>5,120
	gp70	<10	512	80
	p12	<10	60,000	>5,120
anti-R-MuLV p12	—	1,280	<10	5,120
	gp70	1,280	<10	5,120
	p12	<10	<10	<20

* Absorptions were performed by incubation of 0.005 ml sera with 100 μg of the appropriate purified protein in 0.05 ml 0.01 M Tris-HCl, pH 7.8, 1.0 mM EDTA, 0.4% Triton X-100, 0.05 M NaCl for 1 h at 37°C and 18 h, overnight. Sera were subsequently clarified by centrifugation at 100,000 *g* for 2 h.

‡ Immunoprecipitation of ^{125}I-labeled viral proteins and titration of serum blocking activity was performed as described in the legend to Table VI.

Discussion

The results of the present study establish the presence of natural cytotoxic reactivity of spleen lymphocytes from BALB/c mice against syngeneic or allogeneic target cells infected with an endogenous ecotropic type-C virus (B/c-MuLV) or with R-MuLV. The virus-specific nature of the spleen cell cytotoxicity was demonstrated by the lack of reactivity with virus-free syngeneic or allogeneic cells. Low level but significant reactivity of the lymphocytes against a feline cell infected with FeLV may reflect

cross-reactive antigenic determinants shared by the major structural glycoproteins of MuLV and FeLV (31). Type-C virus infection of cells has been shown to confer antigenic and immunogenic changes to the cell that are due in part to structural proteins of the virus but also to nonvirion antigens that are expressed in less immunogenic form in homologous noninfected cells (19, 32). In an attempt to rule out the possibility that the natural killer cell activity observed was due to reaction with nonvirion antigens in the infected target cells, competition assays with concentrated virus were performed. The concentrated virus clearly competed with the target cells for the lymphocyte reactivity confirming the latter's virus-specific reactivity (unpublished observations).

The naturally occurring cytotoxic activity of spleen lymphocytes was not evident in mice lacking isolatable ecotropic virus, and with aging, the incidence of isolatable virus was found to rise progressively, as did the natural cellular cytotoxic reactivity. The close correlation between these two activities suggested strongly that the natural cellular cytotoxicity represents a specific response to endogenous type-C virus expression in the host. Natural cell-mediated cytotoxicity has been previously reported in several mouse strains, including the BALB/c mouse (6–9). This cytotoxic reactivity was inhibited by competition with unlabeled type-C virus expressing target cells (6, 8, 33), however, not all type-C virus-infected cells tested gave positive results in the inhibition assay and virus-free cell lines were also inhibitory (33). These latter studies involved mice aged 40 wk or younger and virus-infected, allogeneic target cells. Mice of this age in our studies did not exhibit a virus-specific cellular immunity but the younger mice did react with noninfected allogeneic target cells; thus, it is felt that allogeneic reactivity cannot be excluded in these previous studies (6, 8). Of interest in the present report was the finding that mice in the older age group, unlike the young group, failed to react with virus-negative allogeneic target cells.

A preliminary characterization of the effector lymphocytes mediating the cytotoxic reactivity is described above. Carbonyl iron treatment with magnetic separation revealed that the activity was not due to, nor dependent on, macrophages. The effector cells failed to adhere to nylon columns nor were they removed by EA or EAC monolayers and thus had no surface receptors for immunoglobulins or complement. This activity was not changed by anti-θ serum and complement revealing that they were devoid of the theta antigen surface marker. With respect to all of these characteristics, the effector cells were similar to previously described N cells (7, 9). The effector cells were clearly different from the cells that mediated anti-MSV cytotoxicity because the latter activity was clearly abrogated by anti-θ serum and complement. Also, being devoid of receptors for immunoglobulins, they were distinguishable from cells mediating antibody dependent cellular cytotoxicity (34–37).

The finding of viral specific cell-mediated and humoral immunity cooccurring in the same individual animal led to the question of their respective roles and interactions in the in vivo immunity of the host. Sera from normal aging mice exhibiting antiviral activity as measured by cytotoxicity assays as well as immune sera with defined antiviral activity were shown to block the lymphocyte mediated cytotoxicity. The viral specific nature of the blocking activity was further demonstrated by absorption experiments with purified viral antigens. Blocking of cellular cytotoxic activity with antisera to target cells is a well established phenomenon (38–41). Previous studies have generally involved analysis of immune lymphocytes and immune sera from mice immunized by tumor allografts. The nature of the antigens of the target cells reactive

with the isoantibody were presumably of the H-2 complex although recent seroepidemiology studies indicate that antiviral activity cannot be ruled out. Blocking of natural killer cell activity with antisera to defined target cell antigens has not been reported. The findings reported here of viral-specific natural killer cell activity and blocking activity of sera with defined antiviral activity provide an opportunity to examine basic immunological effector responses with a well-defined transplantation antigen. The importance of antiviral natural killer cell and blocking activity in the host's immune defenses to its naturally occurring tumors is at present not known but now appears feasible to examine.

Antisera to gp70 and p12, two structural proteins, previously reported to be immunologically reactive at the cell surface in productively infected cells (5, 41) were shown to be highly reactive in the blocking tests. Of interest was the finding that none of the antisera completely blocked the cell-mediated reactivity, suggesting that more than one target antigen was involved in the lymphocyte reactivity. Blocking assays using combinations of antisera are currently under study. Although the findings establish that the cell-mediated activity to virus infected cells involves recognition of gp70 and p12, lymphocyte reactivity with the other viral proteins could not be ruled out as the other viral proteins are probably not immunologically reactive on the cell surface of the particular cells used. It is of interest to try to determine whether the effective immunogen for this cell-mediated reactivity is the endogenous virus itself or cells infected with the endogenous virus. There is some related data suggesting that the latter may be the case. For example, after low dose or hyperimmunization of the mouse with virus (inactivated but retaining high-titered antigenic reactivities), no cell-mediated cytotoxicity has been observed (43), suggesting that a cell-mediated immune response is not evoked by type-C viruses in the mouse. The data reporting cell-mediated immunity to type-C viruses all involve live replicating virus and thus possibly involve infected cells rather than virions as the immunogen (19, 44, 45). Further, the mouse has been shown to be tolerant to the structural components of the virus that are produced by the *gag* gene (1), probably a result of early embryonic expression. Thus, the reactivity to p12 may be induced more readily by p12 active at the cell surface than the p12 contained in virions. This possibility is supported by the fact that recent studies have indicated the expression of a glycosylated *gag* gene-coded precursor polyprotein on the surface of leukemia cells in an immunologically-altered form (46, 47). The importance of clarifying these mechanisms for the induced response is clear when one notes that virtually all of the naturally occurring tumors of two strains of mice contain this viral structural protein (4).

Summary

The natural immune response in mice to their endogenous type-C viruses involves a complex interaction between cellular and humoral immune mechanisms. The virus-specific immune reactivities are a function of age and appear only subsequent to endogenous virus expression. Cellular immune activity was found to reside in a population of lymphocytes that were characterized as natural killer cells based on their absence of theta surface antigens or immunoglobulin or complement receptors. Cellular and humoral virus-specific immune responses co-occur in the same animal and pretreatment of virus-positive target cells with sera from virus-positive aging mice is capable of partially blocking the cytotoxic activity of reactive lymphocytes. The

blocking activity of sera from individual mice increases as a function of age and endogenous virus expression and is highly correlated with the virus-specific complement-dependent cytotoxic activity of these sera. Mouse sera, whether naturally immune or immune as a result of hyperimmunization with type-C virus, exhibit blocking activity that can be removed by absorption with purified type-C virus or purified viral glycoprotein (gp70) but not by absorption with noninfected syngeneic cells. High-titered and highly specific antisera directed against certain individual R-MuLV structural proteins reveal blocking activity. Monospecific antisera to gp70 and p12 exhibited high-titered blocking reactivities which are absorbable by the respective purified proteins. Blocking activity of antisera directed against other viral structural proteins could not be excluded with certainty. These findings raise the possibility that immunity in the mouse to endogenous type-C virus or virus-infected cells involves competition between serum-blocking activity and natural-killer cell activity and further provides a unique model system for studying the mechanism of action of blocking antisera known to have monospecific reactivity against defined and purifiable transplantation antigens.

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