THE GENERATION AND SPECIFICITY OF CYTOTOXIC T CELLS RAISED AGAINST SYNGENEIC TUMOR CELLS BEARING AKR/ GROSS MURINE LEUKEMIA VIRUS ANTIGENS*

By WILLIAM R. GREEN,‡ ROBERT C. NOWINSKI, and CHRISTOPHER S. HENNEY

From the Basic Immunology and Tumor Virology Programs, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104

On the basis of serologically defined cell-surface antigens, virally associated murine leukemias have been classified into two groups: (*a*) leukemias induced by Friend, Moloney, and Rauscher (FMR+) murine leukemia viruses (MuLV)[1] that display a common FMR antigen complex, and (*b*) leukemias that display Gross cell-surface antigen (GCSA+). The latter include those induced by AKR/Gross MuLV and those occurring spontaneously in high leukemic strains such as AKR (1–5).

Recently, it has been observed that mice can generate a cell-mediated cytotoxic response to syngeneic MuLV-induced tumor cells (6–11). The responses to tumors induced by the Moloney leukemia/sarcoma virus complex have been particularly well studied (reviewed in reference 6). Cytotoxic T cells have been raised that lyse a variety of syngeneic tumor cells bearing FMR antigens (7, 8). As cytotoxic T cells stimulated by infection of mice with Friend, Moloney, or Rauscher MuLV reciprocally lysed a variety of syngeneic FMR+ GCSA−, but not syngeneic FMR− GCSA+ or allogeneic FMR+ target cells, it appeared that these effector cells were H-2 restricted and possibly directed against common FMR antigens (7, 8). The cytotoxic response to FMR+ tumors may not invariably be H-2 restricted (9, 10), however, and can apparently be specifically directed against Moloney, but not Rauscher or Friend, virion antigens (11).

In contrast to the well-documented descriptions of effector cells directed against FMR tumors, evidence suggesting that murine cytotoxic cells can be generated to syngeneic tumors bearing GCSA is rather limited. The latter are associated primarily with spontaneous leukemias, and, in general, these are less antigenic to the host than are leukemias induced by the exogenous FMR viruses (2, 12). However, an immune response to such tumors might be anticipated because resistance to Gross virus-induced leukemia is governed in part by a locus (Rgv-1) that maps in the same region (K-I of H-2) as that which controls immune responsiveness (13–16). In further support of this hypothesis, studies of humoral immunity in mice indicate that immune

* Supported by grants AI 15384 and CA 24537 (to Christopher S. Henney) and CA 18074 (to Robert C. Nowinski) from the National Institutes of Health.

‡ Recipient of Fellowship 1 F32 CA 05988-01 from the National Cancer Institute.

[1] *Abbreviations used in this paper:* E/T, effector to target ratio; FCS, fetal calf serum; FMR, Friend, Moloney, Rauscher murine leukemia viruses; GCSA, Gross cell-surface antigen; HBSS, Hanks' balanced salt solution; MuLV, murine leukemia virus; NK, natural killer; PEC, peritoneal exudate cells.

responsiveness to the viral envelope proteins gp70 and p15(E) of endogenous MuLV is coded in the H-2 region (17–18).[2]

Cytotoxic T cells have been raised by repetitive in vitro stimulation with AKR MuLV gp71 and were found to preferentially lyse a virus-producing AKR tumor line when compared with an AKR embryo fibroblast line (19). In another study, relatively weak cytotoxic activity to an AKR tumor line developed in several semisyngeneic F_1 mice after immunization with this tumor (20).

In the present study, we describe initial experiments aimed at determining whether the ability to generate cytotoxic activity against syngeneic GCSA+ tumors correlates with Rgv-directed resistance to leukemogenesis. As a first approach, we have examined the ability of C57BL/6 mice of resistant Rgv genotype to generate cytolytic cells specific for the syngeneic tumor E♂G2, which was originally induced by Gross virus and is the prototype cell for the definition of GCSA antigen (3, 4). A scheme is described whereby such cells were produced by allogeneic in vivo priming followed by secondary in vitro challenge with viral antigen-positive tumors of H-2^b histocompatibility type.

Materials and Methods

Mice. 6- to 8-wk-old male C57BL/6, CBA, and AKR mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). DBA/2 mice were obtained from the Fred Hutchinson Cancer Research Center central animal facilities. Breeding pairs for the AKR·H-2^b congenic line were kindly provided by Dr. E. Boyse (Sloan-Kettering Institute, New York).

Tumor Cells. A spontaneous leukemia cell line derived from an AKR mouse (AKR SL3) was maintained by weekly passage of leukemic spleen cells into AKR mice. These cells were used for in vivo immunization of C57BL/6 mice. In addition, a spontaneous leukemia which arose in an AKR·H-2^b congenic mouse (AKR·H-2^b SL1) and a tumor of C57BL/6 origin (E♂G2) originally derived by infection with Gross virus were similarly maintained in vivo by passage into C57BL/6 mice. These three tumor cell types were also passaged in vitro for use as target cells in ^{51}Cr-release assays or as stimulator cells. EL4 lymphoma (H-2^b) and P815-Y mastocytoma (H-2^d) cells were maintained by serial passage of ascites fluid in syngeneic mice (C57BL/6 or DBA/2, respectively).

The presence of the MuLV-encoded proteins gp70 and p30 on tumor cells was determined by complement-mediated lysis of ^{51}Cr-labeled target cells in microcytotoxicity assays. Target cells were incubated with various dilutions of goat anti-Rauscher gp70 serum, goat anti-AKR p30 serum, or with a rabbit antiserum raised against EL4 cells (21). In general, these goat antisera directed against MuLV proteins identify primarily group-specific antigens. In all cases, exposure to antiserum was for 10 min at room temperature. Rabbit serum was then added at a final concentration of 1:40, and the mixtures further incubated for 50 min at 37°C. At the end of this period, the cells were briefly centrifuged (300 *g*, 30 s), and a portion of the cell-free supernate assessed for its ^{51}Cr content. Percent specific cytolysis was defined as

$$\frac{x-y}{z} \times 100;$$

where x = ^{51}Cr released from target cells incubated with antibody and complement, y = ^{51}Cr released from target cells incubated with medium alone, and z = ^{51}Cr released from target cells after they have been frozen and thawed through three cycles (~ 80% of total). As stated in the legend to Table 1, the percent specific cytolysis observed when target cells were incubated with complement in the absence of added antibody was, with a single exception, ≦ 5%.

Effector Cells. Cytotoxic cells were generated in vivo by intraperitoneal immunization of

[2] Nowinski, R. C., M. Brown, T. Doyle, and R. L. Prentice. Genetic and viral factors influencing the development of spontaneous leukemia in AKR mice. Submitted for publication.

C57BL/6 mice with 10^7 viable AKR SL3 cells. Peritoneal exudate cells (PEC), obtained 8 or more d later, were collected by two washes of the peritoneal cavity with Hanks' balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, N.Y.) containing 1 U/ml sodium heparin (A. H. Robins Co., Richmond, Va.). After three washes in HBSS, the number of viable cells was determined by erythrocin B dye exclusion, and the cells resuspended in RPMI-1640 (Microbiological Associates, Walkersville, Md.) containing 10% heat-inactivated (56°C, 30 min) fetal calf serum (FCS, Grand Island Biological Co.), 100 U/ml penicillin and 100 μg/ml streptomycin. This mixture will subsequently be referred to as assay medium. Single cell suspensions of spleens were made, and contaminating erythrocytes lysed by treatment with 0.83% NH_4Cl. After removal of erythrocyte debris by brief centrifugation (300 *g*, 10 s), spleen cells were washed, counted, and suspended in assay medium.

In vitro generated cytotoxic cells were obtained by culture of spleen cells from normal or in vivo primed C57BL/6 mice in assay medium further supplemented with 100 μg/ml gentamicin (Schering Corporation, Kenilworth, N.J.) and 2 mM L-glutamine (Grand Island Biological Co.) (supplemented medium). Spleen cells (10^7) were cultured alone or together with 10^6, 2×10^5, or 4×10^4 irradiated (3,000 rad, ^{137}Cs irradiator) or mitomycin C- (Sigma Chemical Co., St. Louis, Mo.) treated (100 μg/ml, 37°C, 45 min) tumor cells in individual wells of Falcon 3008 multiwell tissue culture plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) in a total volume of 1.5 ml. After 5–7 d of culture (37°C, 95% air, 5% CO_2), the cells were harvested, washed, counted, and suspended in assay medium for use in cytotoxicity assays.

Cytotoxicity Assays. Tumor cells or spleen cells previously cultured (3–4 d, 37°C, 95% air, 5% CO_2) in supplemented medium containing 10 μg/ml *Escherichia coli* lipopolysaccharide (Difco Laboratories, Detroit, Mich.) were labeled with ^{51}Cr for use as target cells in microcytotoxicity assays as previously described (22). Briefly, 10^4 ^{51}Cr-labeled target cells and varying numbers of effector cells were centrifuged together (300 *g*, 30 s) and then co-incubated in a total volume of 0.2 ml. After 4.5 h at 37°C, the cells were again centrifuged, and an aliquot of cell-free supernate removed for assessment of its ^{51}Cr content. The percent specific lysis was defined as

$$\frac{x-y}{z} \times 100;$$

where x = ^{51}Cr released from target cells incubated with effectors, y = ^{51}Cr released from target cells incubated with medium alone, and z = ^{51}Cr released from target cells after they have been frozen and thawed through three cycles (~ 80% of total).

Spontaneous ^{51}Cr release was that amount released when target cells were incubated with medium alone, expressed as a percentage of the amount released by freeze-thawing.

In "cold-target" competition assays, unlabeled target cells were also included in the reaction mixtures at ratios between 1:1 and 30:1 unlabeled to ^{51}Cr-labeled target cells.

Antisera Treatment of Effector Cells. In some experiments, effector cells were preincubated, just before assessment of their cytotoxicity activity, with antisera (10^7 cells/ml, 25°C, 45 min). After centrifugation, treated cells (or untreated controls) were further incubated in a 1:20 dilution (in RPMI-1640) of rabbit serum as a complement source (10^7 cells/ml, 37°C, 45 min). At the end of this period, the effector cells were washed three times, counted, and resuspended in assay medium at viable cell densities equivalent to those of untreated preparations. The AKR anti-Thy 1.2 serum used was a gift of Dr. G. Klimpel, Fred Hutchinson Cancer Research Center (23). Anti-Lyt 1.2 serum was a gift of Drs. K-E. and I. Hellstrom, Fred Hutchinson Cancer Research Center. The anti-Lyt 2.2 serum was prepared by the method of Shiku et al. (24). The specificities of both anti-Lyt sera have previously been reported (25).

Results

Generation of Cytotoxic Effector Cells against a Syngeneic AKR/Gross Virus-induced Tumor. We were unable to induce significant cytotoxic activity by immunization with up to 10^8 irradiated E♂G2 cells, even when cells from such primed animals were subsequently restimulated with E♂G2 cells in vitro (data not shown). Consequently, C57BL/6 mice were immunized with 10^7 cells of an established allogeneic ($H\text{-}2^k$)

TABLE I
Tumor Cells

Tumor	Inducing agent	Mouse strain	H-2 haplotype	Virus-associated cell surface antigens*	
				gp70	p30
SL3	Spontaneous leukemia	AKR	k	+++	++
E♂G2	Gross virus	C57BL/6	b	+++	+++
SL1	Spontaneous leukemia	AKR·H-2^b	b	++	++
EL4	Dimethylbenzanthracene	C57BL/6	b	±	−
P815Y	Methylcholanthrene	DBA/2	d	−	−

* The presence of the gp70 and p30 proteins of MLV on these tumor cells was determined by the ability of specific antisera and complement to lyse the cells (see Materials and Methods). A range of dilutions of each antiserum was used; the values of positivity presented are the values of percent specific cytolysis obtained with 1:900 anti-gp70 and 1:300 anti-AKR p30 as follows: −, 0–10% lysis; ±, 10–20%; +, 20–40%; ++, 40–70%; and +++, 70–100%. These concentrations of antisera were selected because they were the highest dilutions that caused the maximum amount of lysis of the positive cells. Greater than 50% lysis of all cell types tested was observed when a 1:900 dilution of a rabbit anti-EL4 cell serum and complement was used, indicating that all were susceptible to antibody and complement attack. The values for percent spontaneous release (cells incubated with medium alone) were all ≦ 10%. The values for percent specific lysis when target cells were incubated with complement but not antibody were ≦ 5% except for P815 cells which was ≦ 10%.

leukemic line (AKR SL3) which bears AKR/Gross viral antigens (Table I). It was argued that a response directed against virus-associated antigens common to both the allogeneic (AKR SL3) and syngeneic (E♂G2) tumors might then be detected by employing E♂G2 as a target cell.

When spleen or PEC were harvested 10 d after injection of AKR SL3 cells and tested in short-term ^{51}Cr-release assays, cytotoxic activity was observed against the immunizing allogeneic cell but not against E♂G2 cells (Table II A). In a typical experiment, PEC preparations contained much more activity against AKR SL3 than did spleen cell preparations (Table II A). Furthermore, as both PEC and spleen effector cells lysed AKR SL3 but not E♂G2 (Table II A), EL4 (H-2^b), or P815 (H-2^d) cells (data not shown), it seemed likely that only a cytotoxic response directed against alloantigens had been induced.

In contrast, when spleen cells from the same primed mice (10 d after 10^7 AKR SL3 cells) were additionally cultured in vitro with mitomycin C-treated E♂G2 cells, substantial lytic activity toward E♂G2 developed (Table II B). Strikingly, such restimulation did not induce effector cells capable of lysing AKR SL3 cells. Unprimed spleen cells cultured with E♂G2 cells never generated significant lytic activity (Table II B). On the other hand, restimulation in vitro of AKR SL3-primed spleen cells with the homologous tumor (AKR SL3) led to vigorous activity directed exclusively toward AKR SL3 and thus presumably directed to alloantigen. Unprimed C57BL/6 spleen cells cultured with AKR SL3 cells under the same conditions generated less activity toward AKR SL3. It thus appeared that both in vivo priming with allogeneic cells and in vitro restimulation with syngeneic cells were necessary to induce the development of lytic activity to the syngeneic tumor.

The kinetics of in vivo priming with AKR SL3 cells were studied by immunizing each of a large group of C57BL/6 mice with 10^7 AKR SL3 cells. After 8–30 d, spleen cells were cultured alone or with AKR SL3 or E♂G2 stimulator cells for an additional

TABLE II

Generation and Specificity of C57BL/6 Cytotoxic Effector Cells

	Stimulation*		Effector cells	E/T ratio	Percent specific lysis	
					EδG2(H-2^b)	SL3(H-2^k)
A.	1° in vivo					
	None		Spleen cells	200:1	−0.8	−4.4
			PEC	50:1	−2.3	−2.8
	SL3		Spleen cells	200:1	1.2	13.0
			PEC	50:1	0	60.3
B.	1° in vivo	2° in vitro				
	None	None	Spleen cells	100:1	6.7	10.0
		SL3	Spleen cells	100:1	5.9	38.9
		EδG2	Spleen cells	100:1	7.0	6.5
	SL3	None	Spleen cells	100:1	8.4	11.9
		SL3	Spleen cells	100:1	6.8	81.8
		EδG2	Spleen cells	100:1	65.4	6.4

C57BL/6 mice were injected with 10^7 SL3 cells and killed 10 d later. The lytic activity of PEC and spleen cells was either measured immediately (part A) or after 5 d of in vitro culture (part B). Although only one E/T cell ratio is presented for simplicity in part B, two other ratios were employed for each cell preparation. The average values for spontaneous release were 3.3 or 1.8%/h (SL3) and 2.8 or 3.7%/h (EδG2) for experiments A and B, respectively.

5 d before assessment of lytic activity. At some of the time-points, PEC and spleen cells were assessed for activity without in vitro restimulation.

Although activity against EδG2 cells was never observed after in vivo priming alone, spleen cells harvested 8–12 d after injection of AKR SL3 generated a subtantial amount of activity toward EδG2 target cells when restimulated with EδG2 (Fig. 1). Interestingly, spleen cells harvested 14–30 d after priming with AKR SL3 cells were consistently less responsive to EδG2 stimulation than were spleen cells taken 10 d after priming. In contrast, spleen cell populations harvested 14–30 d after AKR SL3 priming still responded vigorously to challenge with AKR SL3 cells in vitro (Fig. 1). Thus the ability to respond to allogeneic tumors appeared to persist longer than that to syngeneic cells (Fig. 1).

Several parameters of in vitro culture were examined for their effect on the generation of activity toward EδG2, and the following observations were made (data not shown):

(*a*) The response of AKR SL3-primed cells to EδG2 stimulation was rarely seen after 3 d of culture, peaked after 5 or 6 d, and remained undiminished through day 7.

(*b*) Both mitomycin C-treated and irradiated (3×10^3 rad) EδG2 were able to stimulate a syngeneic cytotoxic response.

(*c*) Both in vivo and in vitro passaged EδG2 cells were suitable as stimulator cells.

(*d*) Several lots of FCS were found to support the development of lytic activity against EδG2, and the presence of 5×10^{-5} M 2-mercaptoethanol did not augment such activity.

Characterization of Effector Cells. To determine whether the cytotoxic cells directed

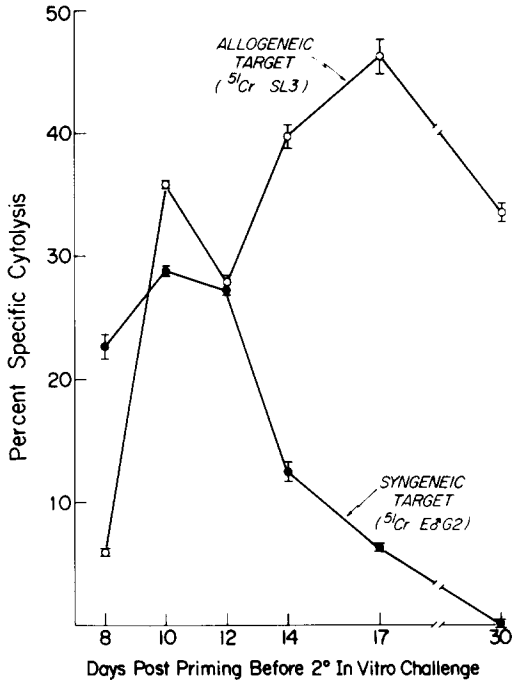

FIG. 1. Development of the ability of SL3-primed spleen cells to generate cytotoxic cells after in vitro restimulation. 18 C57BL/6 mice were injected with 10^7 SL3 cells, and 3 mice were killed on each of the indicated days after immunization. Spleen cells were then cultured an additional 5 d in vitro by themselves or in the presence of mitomycin C-treated syngeneic EδG2 or allogeneic SL3 cells at various responder to stimulator cell ratios (10:1, 50:1, or 250:1). Maximum activity against the homologous target at an effector to target (E/T) ratio of 100:1 for effector cells restimulated with EδG2 cells (●), and at an E/T ratio of 33:1 for effector cells restimulated with SL3 cells (○), is plotted. Because the amount of allogeneic activity observed in parallel cultures of SL3-primed spleen cells incubated without restimulation was often considerable, this amount of activity has been subtracted from the indicated activities against SL3 cells. The error bars indicate the range of the actual duplicate determinations of percent specific lysis.

against AKR SL3 or EδG2 were of thymic origin, cells of each specificity were sequentially preincubated with various dilutions of anti-Thy 1.2 antiserum and complement just before assessment of lytic activity. As can be seen from the results of a representative experiment (Fig. 2), cytotoxic cells directed against either EδG2 or AKR SL3 were essentially identical in their susceptibility to anti-Thy 1.2 and complement. We thus consider that the effector cells directed against EδG2, like those against AKR SL3, are T cells.

It has been reported that C57BL/6 effector cells directed against syngeneic tumors are, like their precursors, of the Lyt 1+2+3+ phenotype (26), thus distinguishing them from C57BL/6 effector cells directed against H-2^d alloantigens which are Lyt 2+3+ but Lyt 1− (29). We therefore considered it of interest to determine the display of Lyt antigens on the effector cells described in Table II. In the experiment shown in Table III, the cytotoxic activity of C57BL/6 effector cells raised against EδG2 tumor cells was completely abrogated by the action of anti-Lyt 2.2 and complement, as was that of cytotoxic cells generated against allogeneic cells. Furthermore, and in keeping with the report of Shiku et al. (26), the effector cell activity

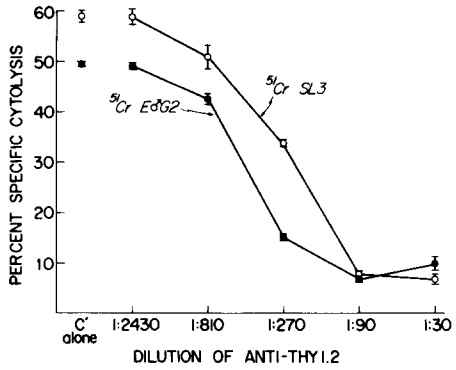

FIG. 2. Susceptibility to anti-Thy 1.2 serum and complement of C57BL/6 effector cells raised against either syngeneic (EδG2) or allogeneic (SL3) tumors. SL3-primed C57BL/6 spleen cells were restimulated with and tested against SL3 cells (○) or restimulated with and tested against EδG2 cells (●). Untreated effector cells or effector cells preincubated with various dilutions of anti-Thy 1.2 serum and/or complement (1:20 rabbit serum), were assayed at an effector to target cell ratio of 100:1. The average percent spontaneous release was 2.4%/h for EδG2 and 4.8%/h for SL3 cells. The error bars indicate the range of the actual duplicate determinations of percent specific lysis. SL3-primed spleen cells cultured without restimulation caused 1.1 and 6.5% specific lysis of EδG2 and SL3 cells, respectively.

TABLE III

Lyt Phenotypes of C57BL/6 Cytotoxic T Cells

Stimulation*		Treatment	Percent specific lysis	
1° in vivo	2°in vitro		SL3	EδG2
SL3	SL3	None	58.9	ND‡
		C only	54.1	
		1:20 anti-Lyt 1.2 + C	30.9	
		1:20 anti-Lyt 2.2 + C	7.5	
SL3	EδG2	None	ND	21.1
		C only		21.3
		1:20 anti-Lyt 1.2 + C		2.0
		1:20 anti-Lyt 2.2 + C		−1.3

Spleen cells from C57BL/6 mice injected 11 d earlier with 10^7 SL3 cells were cultured in vitro for 6 d with either irradiated SL3 or irradiated EδG2 cells. The data presented represent the amount of lytic activity observed when either untreated effector cells or those pretreated as indicated were assayed against the stimulating target cell at E/T ratios of 8:1 (anti-SL3 effectors vs. SL3) or 75:1 (anti-EδG2 effectors vs. EδG2). Additional E/T ratios were also tested. The average values for spontaneous release were 2.9%/h for EδG2 cells and 7.0%/h for SL3 cells. The amount of anti-Lyt serum employed was empirically determined to be the highest dilution that would ablate either alloantigen-primed cytotoxic cells (anti-Lyt 2.2) or "helper" activity in antibody responses to sheep erythrocytes (anti-Lyt 1.2) (25).

* Not determined.

directed against syngeneic tumor, was ablated by the action of the anti-Lyt 1.2 serum. The activity against allogeneic target cells was clearly less affected by this reagent. Thus, the cytotoxic T cells raised against EδG2 tumor cells appeared to bear both Lyt 1.2 and Lyt 2.2 alloantigens.

Target Cell Specificity of the Cytotoxic T Cells. As a first approach to defining their

TABLE IV

Cytotoxic Specificity of Restimulated Spleen Cells from SL3-Primed C57BL/6 Mice

Stimulation*		E/T ratio	Percent specific lysis					
			H-2^k		H-2^b			H-2^d
1° in vivo	2° in vitro		SL3	CBA blasts	EδG2	SL1	EL4	P815
SL3	None	50:1	14.5	0.8	0.4	1.7	−2.3	−3.2
		17:1	5.4	−1.5	0.6	0.3	−2.4	−1.6
		6:1	1.6	−5.4	0.2	−0.5	−1.7	−1.1
SL3	SL3	50:1	33.1	18.8	0.6	0.8	−1.4	0.2
		17:1	26.7	7.1	0	0.6	−1.7	−0.8
		6:1	11.9	3.1	0.7	−0.7	−1.4	−0.9
SL3	EδG2	50:1	17.2	2.9	35.3	32.5	−1.0	0
		17:1	6.5	−1.1	21.4	17.2	−1.7	−0.8
		6:1	2.5	−4.8	9.8	5.9	−1.2	−0.2

Spleen cells from C57BL/6 mice injected 10 d earlier with 10^7 SL3 cells were removed and placed in tissue culture for 6 d with or without additional stimulation as shown before assessment of their lytic activity. The average values for spontaneous release were 3.7%/h (SL3), 2.0%/h (EδG2), 2.2%/h (SL1), 2.4%/h (EL4), 10.1%/h (CBA blasts), and 2.5%/h (P815).

specificity, effector cells generated against EδG2 cells were assayed against a wide spectrum of target cells. These included P815 mastocytoma cells of the unrelated H-2^d haplotype, normal CBA (H-2^k) spleen cells stimulated by lipopolysaccharide (CBA blasts), and two additional target cells of H-2^b haplotype: EL4 and AKR·H-2^b SL1, the latter a cell line derived from a spontaneous leukemia which developed in an AKR·H-2^b congenic mouse. As shown in Table I, EL4 and P815 cells were negative or weakly positive for display of gp70 and p30 viral antigens relative to the highly positive AKR SL3, EδG2, and AKR·H-2^b SL1 cells.

In a representative experiment (Table IV), restimulation of AKR SL3-primed spleen cells with EδG2 caused the development of lytic activity only to EδG2 and AKR·H-2^b SL1 target cells. Interestingly, EL4 cells, though of H-2^b haplotype and weakly positive for gp70 display, were not lysed. Competition experiments were also carried out with these tumor cells. In these studies, effector cells induced by AKR SL3 priming followed by restimulation with EδG2 were tested against ^{51}Cr-labeled EδG2 cells in the presence of varying numbers of unlabeled target cells. Although EδG2 and AKR·H-2^b SL1 cells effectively inhibited the lysis of EδG2 cells, AKR SL3, P815, and EL4 cells and CBA blasts were not inhibitory (Fig. 3 b).

There was some lytic activity against AKR SL3 in EδG2 restimulated cultures, but a comparable amount of activity was observed in cultures of AKR SL3-primed spleen cells incubated without further stimulation (Table IV). This activity presumably represented a population of cytotoxic cells directed against alloantigen because the activity was augmented by restimulation with AKR SL3 cells and was also directed against CBA blasts (Table IV). Because CBA blasts were lysed much less efficiently than AKR SL3 cells, competition experiments were performed to determine whether

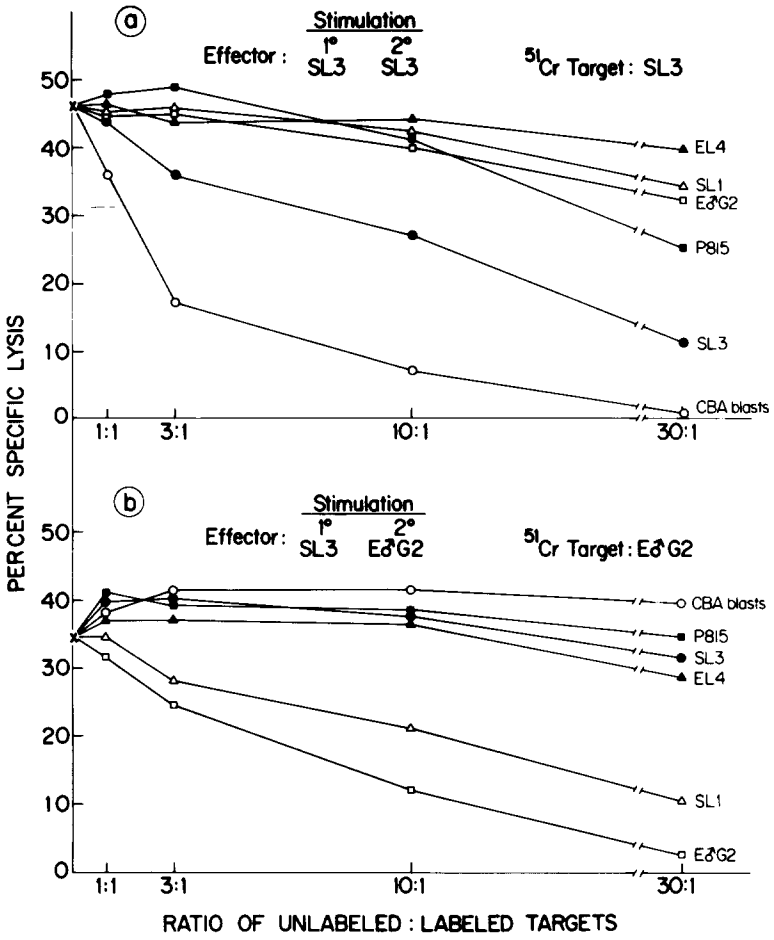

FIG. 3. Competitive inhibition of C57BL/6 effector cells raised against SL3 or EδG2 cells. In Fig. 3 a C57BL/6 effector cells raised by restimulation of SL3-primed spleen cells with SL3 were assayed against ^{51}Cr-labeled SL3 cells at an E/T ratio of 20:1 in the presence or absence of the indicated numbers of unlabeled target cells. The percent specific lysis of SL3 cells by primed but non-restimulated spleen cells was 18.4%. The average percent spontaneous release was 6.5%/h. In Fig. 3 b C57BL/6 effector cells raised by restimulation of SL3-primed spleen cells with EδG2 cells were assayed against ^{51}Cr-labeled EδG2 cells at an E/T ratio of 50:1 in the presence or absence of unlabeled cells. The percent specific lysis of EδG2 cells by primed but non-restimulated spleen cells was 0.4%. The average percent spontaneous release was 2.0%/h.

effector cells raised by secondary stimulation with AKR SL3 cells were only directed against H-2^k alloantigens. When the lytic activity of AKR SL3-primed and restimulated effector cells was assessed against labeled AKR SL3 cells in the presence of varying numbers of unlabeled target cells, only AKR SL3 and CBA blasts were effective competitive inhibitors of lysis (Fig. 3 a). In contrast to their ability to inhibit effector cells raised against the syngeneic tumor, EδG2 and AKR·H-2^b SL1 cells were not inhibitory. This finding, coupled with the observation that CBA blasts were reproducibly more efficient competitor cells than were AKR SL3 cells, suggested that the predominant lytic activity raised against AKR SL3 cells was directed against H-

TABLE V

Ability of Tumor Cells to Stimulate the Generation of Cytotoxic T Cells from SL3-Primed C57BL/6 Spleens

Stimulation*		E/T ratio	Percent specific lysis		
1° in vivo	2° in vitro		SL3	EδG2	SL1
SL3	None	100:1	39.3	3.0	0.5
		33:1	20.9	0.1	0
		11:1	6.8	−0.3	−0.2
SL3	SL3	100:1	67.8	2.8	0.4
		33:1	55.9	0.9	−1.0
		11:1	29.2	0.7	−0.9
SL3	EδG2	100:1	37.5	86.9	42.8
		33:1	22.1	71.5	31.4
		11:1	10.2	41.3	20.3
SL3	SL1	100:1	39.4	82.1	42.7
		33:1	25.9	48.9	26.6
		11:1	10.0	20.3	10.8
SL3	EL4	100:1	46.1	1.9	2.1
		33:1	29.2	1.1	−0.3
		11:1	12.0	0.1	0.4

Spleen cells from C57BL/6 mice injected 9 d earlier with 10^7 SL3 cells were removed and placed in tissue culture for 6 d with or without additional stimulation as shown before assessment of their lytic activity. Three responder to stimulator cell ratios (10:1, 50:1, and 250:1) were employed for each type of 2° stimulation, and the data depicted for that giving the highest amount of lysis (generally 50:1). The average values for spontaneous release were 4.9%/h (SL3), 2.8%/h (EδG2), and 2.6%/h (SL1).

2^k alloantigens. Thus, in this immunization regime there was no evidence for the presence of activity against viral or tumor-specific antigens.

Specificity of Stimulator Cells in the Generation of Lytic Activity to EδG2 and AKR·H-2^b SL1 Targets. To determine whether the susceptibility of tumor cells to attack by effector cells raised against EδG2 correlated with their ability to serve as stimulator cells in the generation of these cytotoxic T cells, irradiated AKR·H-2^b SL1 or EL4 cells were co-cultured with AKR SL3-primed spleen cells. Three responder to stimulator cell ratios were used (10:1, 50:1, and 250:1). Irradiated EδG2 and AKR SL3 cells were used in parallel cultures. In a typical experiment, lytic activity toward EδG2 and AKR·H-2^b SL1 cells developed only in cultures in which either of these two cells were included (Table V). AKR·H-2^b SLI cells appeared to be nearly as efficient stimulator cells as EδG2 cells, although they were usually somewhat less susceptible as target cells (Table V, Figs. 3 b and 4). Consistent with their inability to serve as targets, EL4 cells were also ineffective as stimulator cells. As previously observed (Tables II and IV), there was a comparable amount of activity to AKR SL3 in all cultures except those to which AKR SL3 stimulator cells had been added. In an experiment not shown, P815 cells were unable to restimulate AKR SL3-primed spleen cells to develop into effector cells capable of lysing EδG2 cells although significant

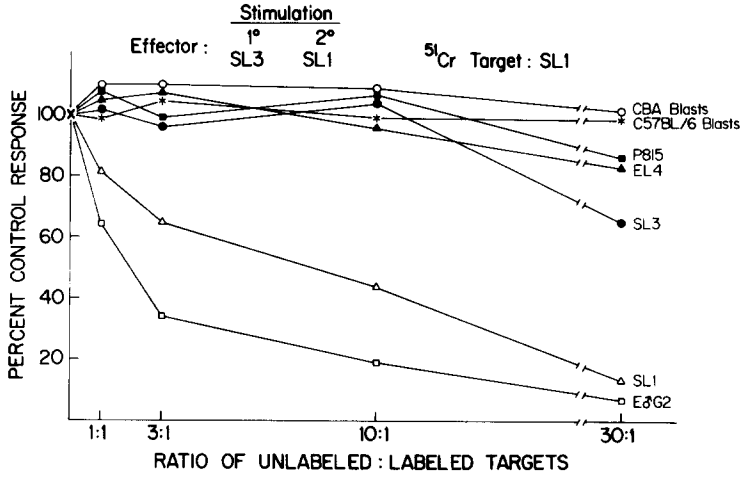

FIG. 4. Competitive inhibition of C57BL/6 effector cells raised against SL1 cells. Fig. 4 is a composite of two separate experiments in which effector cells raised by restimulation of SL3-primed spleen cells with SL1 cells were assayed against ^{51}Cr-labeled SL1 cells at an E/T ratio of 100:1 in the presence or absence of the indicated numbers of unlabeled target cells. The percent specific lysis of SL1 by these effector cells was 17.8 and 29.6% in two separate experiments. SL3-primed spleen cells cultured without restimulation caused −0.7 or 3.4% specific lysis of SL1 cells. The average percent spontaneous release was 2.2%/h in one experiment and 3.7%/h in the other.

lytic activity against P815 cells did develop.

Specificity of Effector Cells Induced after Restimulation with AKR·H-2^b SL1. The possibility that the lytic activity generated by restimulation of AKR SL3-primed spleen cells with AKR·H-2^b SL1 cells might be entirely directed against specificities shared by E♂G2 and AKR·H-2^b SL1 cells was addressed (Table V). Inasmuch as the SL1 line was derived from an AKR·H-2^b congenic mouse, it seemed possible that lytic activity might develop against AKR alloantigens other than those encoded by H-2. However, restimulation of AKR SL3-primed spleen cells with AKR·H-2^b SL1 cells did not augment the lysis of AKR SL3 cells, which should be a measure of such minor alloantigen activity (Table V). The presence of significant activity against AKR SL3 in all cultures of AKR SL3-primed spleen cells, however, might have obscured the detection of such activity. To address this problem in a more sensitive manner, effector cells obtained by restimulation with AKR·H-2^b SL1 cells were assayed against ^{51}Cr-labeled AKR·H-2^b SL1 cells in the presence of a variety of unlabeled cells including AKR SL3. In a composite of two separate experiments (Fig. 4), only slight inhibition by AKR SL3 cells was observed and then only at the highest cell number used. Furthermore, E♂G2 cells were even more efficient than homologous AKR·H-2^b SL1 cells as unlabeled competitors. Taken together, these findings suggest that the amount of lytic activity that develops against "minor" AKR alloantigens when AKR·H-2^b SL1 cells are used to restimulate AKR SL3-primed populations is small. Other target cells including lipopolysaccharide-"blasts" of C57BL/6 spleen cells and EL4 cells were ineffective competitor cells, further indicating that the display of H-2^b alloantigens was not sufficient for recognition.

Discussion

In the present study we have described the production of C57BL/6 cytotoxic T

cells against tumor cell lines of homologous histocompatibility type which display AKR/Gross viral antigens. The following findings seem pertinent in addressing the specificity of these cytotoxic cells:

(*a*) In vivo priming with allogeneic AKR/Gross viral antigen-positive cells (AKR SL3) was necessary for their generation (Table II).

(*b*) In vitro restimulation with cells of H-2^b haplotype strongly positive for viral antigens (E♂G2 or AKR·H-2^b SL1) was also necessary for their generation; AKR SL3 cells or weakly gp70-positive EL4 (H-2^b) cells were ineffective (Tables II, IV, and V).

(*c*) The effector cells raised in this manner recognized only E♂G2 and AKR·H-2^b SL1 cells as target cells, not AKR SL3, EL4, or P815 (H-2^d) cells, or CBA (H-2^k) or C57BL/6 (H-2^b) spleen cell blasts (Table IV, Figs. 3 and 4).

Spleen cells were able to respond to E♂G2 stimulation after priming with AKR SL3 cells, but not after priming with large numbers (up to 10^8) of AKR spleen cells, even though the latter lead to a significant allogeneic cytotoxic response (data not shown). These observations imply that AKR SL3, E♂G2, and AKR·H-2^b SL1 cells may share common antigenic specificities against which the cytotoxic cells are directed. Serological studies (Table I) suggest that these specificities might be AKR/Gross virus-encoded products, although "derepressed" or modified cellular-specified antigens could also be candidates. These considerations and the fact that the susceptible AKR·H-2^b SL1 cells, though of H-2^b haplotype, are of the same AKR background as are the insusceptible AKR SL3 cells argue for the possibility that the effector cells are H-2 restricted and directed against AKR/Gross virus-associated antigens.

There are, of course other possible explanations for the observed specificity. It is conceivable, for example, that the AKR SL3 cells do not bear the antigens which the effector cells recognize on E♂G2 and AKR·H-2^b SL1 cells. Thus, even though the allogeneic AKR SL3 cells were able to "prime" for responsiveness to subsequent E♂G2 stimulation, while AKR spleen cells bearing the same alloantigens were not, priming may be a result of a unique ability to AKR SL3 cells to induce a response that dramatically amplifies the ability to respond to weakly immunogenic antigens common to E♂G2 and AKR·H-2^b SL1 cells. These antigens could be confined to E♂G2 and AKR·H-2^b SL1 or, if the cytotoxic cells are indeed restricted, be more ubiquitously distributed.

Formal determination of whether the effector cells are H-2 restricted depends on the definition of the antigens recognized and their introduction into cells of a variety of H-2 haplotypes. As a first approach to the issues of specificity and restriction, we are currently attempting to infect cells of H-2^b haplotype that are not susceptible to effector cells raised against E♂G2 (such as EL4). If we are able to convert insusceptible target cells to susceptible ones by infection with virus preparations from E♂G2 or AKR SL3, we will not only demonstrate that the antigen recognized is virus associated, but will also have a system to address the question of restriction.

The possibility that AKR SL3 cells may release infectious virus in vivo must be considered in light of the observations that priming can be accomplished by injection of AKR SL3 but not by AKR spleen cells or by irradiated E♂G2 cells. Thus, priming may occur by the release of virions that infect host cells and induce the display of antigens recognized on susceptible target cells. Priming by an infectious process is

appealing because in vitro cultured AKR SL3 cells do release infectious virus (28), and because such an infection of host cells is easily reconciled with the postulate H-2 restricted nature of the cytotoxic T cells.

An alternative explanation for the observation that, of the cell types tested, only AKR SL3 has the ability to "prime," is the possibility that initial stimulation by both alloantigens and the target cell antigen is required. Although it is unclear whether dual presentation of target cell antigen and alloantigen is necessary in the present system, there is a precedent for this kind of stimulatory requirement. Zarling et al. (29) have observed that when lymphocytes from leukemic patients in remission are concurrently stimulated with a mixture of autologous leukemic cells and normal allogeneic cells, cytotoxic cells directed against the autologous leukemic cells were produced. The leukemic cells themselves failed to stimulate such activity. Arguing by analogy, one might predict that simultaneous immunization with the syngeneic E♂G2 tumor and allogeneic cells might also prime spleen cells to subsequently respond to E♂G2 stimulation in vitro. These experiments are presently underway. If this proves to be the case, such findings coupled with Zarling's observations might establish general guidelines for inducing cytotoxic responses to syngeneic tumors.

Although the manner in which spleen cells are primed in vivo and the absolute specificity of the cytotoxic cells directed against E♂G2 thus remain unresolved, the nature of these cytotoxic cells is considerably better defined. Effector cells raised against the syngeneic tumor by priming with AKR SL3 and restimulation with E♂G2 cells were as susceptible to the action of anti-Thy 1.2 serum and complement as were effector cells of allogeneic specificity that were also raised by secondary stimulation (Fig. 2). This observation argues that the effector cell that lyses E♂G2 is a T cell rather than a natural killer (NK) cell. The latter, although they have been reported to bear Thy 1 alloantigen, display significantly less of this alloantigen than do T effector cells (30, 31). Additional evidence against the participation of an NK effector cell in the phenomenon reported here includes the following: (*a*) the effector cells described require prolonged in vitro culture for their induction (NK cells are generally considered to be labile under "standard" culture conditions; 31), and (*b*) the effector cells described showed marked target cell specificity (NK cells do not display such stringent preference with respect to either H-2 or tumor cell type; 32). Moreover, AKR SL3 cells, which were not lysed or recognized by the effector cells directed against E♂G2 and AKR·H-2^b SL1 cells (Tables IV and V, Figs. 3 b and 4), were found to be equally or more susceptible than these latter tumors to NK cells of C57BL/6 origin (Green, W., and J. Durdik, unpublished observations).

The lytic activity to syngeneic E♂G2 cells was completely abolished by treatment with either anti-Lyt 1.2 or anti-Lyt 2.2 serum and complement (Table III). These findings are thus compatible with earlier reports of Shiku et al. (26) that C57BL/6 cytotoxic cells directed against syngeneic target cells are of the Lyt 1+2+ phenotype. This conclusion is limited, however, by the observations that in syngeneic situations the precursors of cytotoxic cells may also be of Lyt 1+2+ phenotype, and these precursors may differentiate, during a long-term (24 h or more) lytic assay itself, into Lyt 1-2+ effector cells (26, 33). Even though the duration of cytotoxic assays in the present system was only 4.5 h, this possibility must be considered.

The activity of the cytotoxic T cells raised against H-2^k alloantigens was also completely ablated by treatment with anti-Lyt 2.2 serum and complement but only

partially reduced by anti-Lyt 1.2 serum and complement (Table III). This observation is thus generally consistent with previous findings that cytolytic activity toward alloantigens is much more susceptible to anti-Lyt 2.2 than to anti-Lyt 1.2 serum (27).

Our finding that after appropriate stimulation, C57BL/6 mice have the ability to mount a substantial cytotoxic response against AKR/Gross viral antigen-positive cell lines of H-2^b haplotype may represent an in vitro correlate of the known genetic resistance of these mice to Gross virus-induced leukemia. Further analysis of the ability of other strains of mice will be required to determine whether such responsiveness does, in fact, correlate with the presence of genes at the Rgv-1 or Rgv-2 loci which govern resistance to Gross virus. The possible association of responsiveness with the Rgv-1 locus will be especially interesting because the latter has been mapped in the K-I region of H-2 (13, 14) where a variety of genes governing immune responsiveness have also been located (15, 16). Linkage of cytotoxic responsiveness against AKR/Gross virus-associated antigens to Rgv-1-determined resistance, if found, may thus be a contributing factor in the high incidence of spontaneous leukemia of certain strains of mice such as AKR.

Summary

Efforts were made to generate C57BL/6 cytotoxic effector cells to a syngeneic leukemia (E♂G2) bearing AKR/Gross virus antigens. As we were unable to induce significant cytotoxic activity by immunization with up to 10^8 irradiated E♂G2 cells, even when cells from such primed animals were subsequently restimulated with E♂G2 cells in vitro, C57BL/6 mice were immunized with an allogeneic, virus-producing AKR leukemic cell line (AKR SL3).

Peritoneal exudate cells and, to a lesser degree, spleen cells from these mice showed significant lytic activity toward the immunizing allogeneic tumor but not toward E♂G2. When spleen cells were harvested from animals ≅ 10 d after injection of AKR SL3 and rechallenged in vitro with either E♂G2 or AKR·H-2^b SL1, another tumor that displays AKR/Gross virus antigens, then a vigorous cytotoxic response against E♂G2 and AKR·H-2^b SL1 was obtained.

Effector cells generated by AKR SL3 priming followed by in vitro stimulation with E♂G2 or AKR·H-2^b SL1 lysed only cells of H-2^b haplotype which were strongly positive for the display of serologically detectable AKR/Gross virus antigens. Thus, AKR SL3 cells were not lysed nor were EL4 cells (H-2^b; but only weakly positive for gp70). Cells not bearing the MuLV antigens tested for, such as P815 mastocytoma cells and spleen cell "blasts" from C57BL/6 and CBA (H-2^k) mice, were also insusceptible to attack. The cytotoxic effector cells induced bore Thy 1.2 alloantigen and were of the Lyt 1+2+ phenotype.

Collectively, these findings are consistent with the conclusion that the cytotoxic T cells raised against E♂G2 are directed against AKR/Gross virus-associated antigens and are H-2 restricted. It will be of interest to determine the relevance of such effector cells to the known resistance of the C57BL/6 mouse to AKR/Gross virus-induced leukemia.

The expert technical assistance of Mr. Robert Bick and the typing of the manuscript by Ms. Jan Rothweiler were greatly appreciated.

Received for publication 28 March 1979.

References

1. Gross, L. 1951. Spontaneous leukemia developing in C3H mice following inoculation in infancy with AK leukemic extracts or AK embryos. *Proc. Soc. Exp. Biol. Med.* **76:**27.
2. Old, L. J., and E. Stockert. 1977. Immunogenetics of cell surface antigens of mouse leukemia. *Annu. Rev. Genet.* **17:**217.
3. Old, L. J., E. A. Boyse, and E. Stockert. 1975. The G (Gross) leukemia antigen. *Cancer Res.* **25:**813.
4. Old, L. J., E. A. Boyse, and E. Stockert. 1964. Typing of mouse leukemias by serological methods. *Nature (Lond.).* **201:**777.
5. Old, L. J., and E. A. Boyse. 1965. Antigens of tumors and leukemias induced by viruses. *Fed. Proc.* **24:**1009.
6. Levy, J. P., and J. C. Leclerc. 1977. The murine sarcoma virus-induced tumor: exception or general model in tumor immunology? *Adv. Cancer Res.* **24:**1.
7. Gomard, E., V. Duprez, T. Reme, M. J. Colombani, and J. P. Levy. 1977. Exclusive involvement of H-2D^b or H-2K^d product in the interaction between T-killer lymphocytes and syngeneic H-2^b or H-2^d viral lymphomas. *J. Exp. Med.* **146:**909.
8. Gomard, E., J. P. Levy, F. Plata, Y. Henin, V. Duprez, A. Bismuth, and T. Reme. 1978. Studies on the nature of the cell surface antigen reacting with cytolytic T lymphocytes in murine oncornavirus-induced tumors. *Eur. J. Immunol.* **8:**228.
9. Ting, C. C., and L. W. Law. 1977. Studies of H-2 restriction in cell-mediated cytotoxicity and transplantation immunity to leukemia-associated antigens. *J. Immunol.* **118:**1259.
10. Holden, H. T., S. Landolfo, and R. B. Herberman. 1977. T-cell-dependent reactivity against tumor-associated antigens on allogeneic target cells. *Trans. Proc.* **9:**1149.
11. Enjuanes, L., J. C. Lee, and J. N. Ihle. 1979. Antigenic specificities of the cellular immune response of C57BL/6 mice to the Moloney leukemia/sarcoma virus complex. *J. Immunol.* **122:**665.
12. Nowinski, R. C., S. Emery, and J. Ledbetter. 1978. Identification of an FMR cell surface antigen associated with murine leukemia virus-infected cells. *J. Virol.* **26:**805.
13. Lilly, F. 1966. The inheritance of susceptibility to the Gross leukemia virus in mice. *Genetics.* **53:**529.
14. Lilly, F. 1970. The role of genetics in Gross virus leukemogenesis. *Bibl. Haematol.* **36:**213.
15. Benacerraf, B., and H. O. McDevitt. 1972. Histocompatibility-linked immune response genes. *Science (Wash. D. C.).* **175:**273.
16. McDevitt, H. O., and W. F. Bodmer. 1972. Histocompatibility antigens, immune responsiveness, and susceptibility to disease. *Am. J. Med.* **52:**1.
17. Nowinski, R. C. 1976. Genetic control of natural immunity to ecotropic mouse leukemia viruses: immune response genes. *Infect. Immun.* **13:**1098.
18. Nowinski, R. C., and T. Doyle. 1976. Antibody to murine leukemia virus: genetic control linked to the H-2 locus in PL mice. *J. Immunol.* **117:**350.
19. Lee, J. C., and J. N. Ihle. 1977. Characterization of the blastogenic and cytotoxic responses of normal mice to ecotropic C-type viral gp71. *J. Immunol.* **118:**928.
20. Muerelo, D., B. Deak, and H. O. McDevitt. 1977. Genetic control of cell-mediated responsiveness to an AKR tumor-associated antigen. *J. Exp. Med.* **146:**1367.
21. Green, W. R., Z. K. Ballas, and C. S. Henney. 1978. Studies on the mechanism of lymphocyte-mediated cytolysis. XI. The role of lectin in lectin-dependent cell-mediated cytotoxicity. *J. Immunol.* **121:**1566.
22. Thorn, R. M., J. C. Palmer, and L. A. Manson. 1974. A simplified ^{51}Cr-release assay for killer cells. *J. Immunol. Methods.* **4:**301.
23. Klimpel, G. R., and C. S. Henney. 1978. Demonstration of a macrophage-like suppressor cell that inhibits cytotoxic T cell generation *in vitro*. *J. Immunol.* **120:**563.
24. Shiku, H., P. Kisielow, M. A. Bean, T. Takahashi, E. A. Boyse, H. F. Oettgen, and L. J. Old. 1975. Expression of T-cell differentiation antigens on effector cells in cell-mediated

cytotoxicity *in vitro*. Evidence for functional heterogeneity related to the surface phenotype of T cells. *J. Exp. Med.* **141:**227.

25. Okada, M., G. R. Klimpel, R. C. Kuppers, and C. S. Henney. 1979. The differentiation of cytotoxic T cells *in vitro*. I. Amplifying factor(s) in the primary response are Lyt 1+ cell dependent. *J. Immunol.* In press.
26. Shiku, H., T. Takahashi, M. A. Bean, L. J. Old, and H. F. Oettgen. 1976. Ly phenotype of cytotoxic T cells for syngeneic tumor. *J. Exp. Med.* **144:**1116.
27. Cantor, H., and E. A. Boyse. 1975. Functional subclasses of T lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. *J. Exp. Med.* **141:**1376.
28. Nowinski, R. C., E. F. Hays, T. Doyle, S. Linkhart, E. Medeiros, and R. Pickering. 1977. Oncornaviruses produced by murine leukemia cells in culture. *Virology.* **81:**363.
29. Zarling, J. M., P. C. Raich, M., McKeough, and F. H. Bach. 1976. Generation of cytotoxic lymphocytes *in vitro* against autologous human leukaemia cells. *Nature* (*Lond.*). **262:**691.
30. Herberman, R. B., M. E. Nunn, and H. T. Holden. 1978. Low density of Thy I antigen on mouse effector cells mediating natural cytotoxicity against tumor cells. *J. Immunol.* **121:**304.
31. Wolfe, S. A., D. E. Tracey, and C. S. Henney. 1977. BCG-induced murine effector cells. II. Characterization of natural killer cells in peritoneal exudates. *J. Immunol.* **119:**1152.
32. Tracey, D. E., S. A. Wolfe, J. M. Durdik, and C. S. Henney. 1977. BCG-induced murine effector cells. I. Cytolytic activity in peritoneal exudates: an early response to BCG. *J. Immunol.* **119:**1145.
33. Stutman, O., F. W. Shen, and E. A. Boyse. 1977. Ly phenotype of T cells cytotoxic for syngeneic mouse mammary tumors: evidence for T cell interactions. *Proc. Natl. Acad. Sci. U. S. A.* **74:**5667.