

QUANTITATIVE VARIATIONS IN THE EXPRESSION OF *H-2*
ANTIGENS ON MURINE LEUKEMIA VIRUS-INDUCED TUMOR
CELLS CAN AFFECT THE *H-2*-RESTRICTION PATTERNS OF
TUMOR-SPECIFIC CYTOLYTIC T LYMPHOCYTES*

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In mice, *H-2* antigens coded for by genes in the major histocompatibility complex (MHC)¹ have a directive influence on the specific recognition of syngeneic tumor cells by cytolytic T lymphocytes (CTL) irrespective of the origin of the tumor. Thus, joint recognition of *H-2* and tumor antigens by syngeneic CTL has been reported for tumors induced by murine leukemia viruses (MuLV; 1–6), by Simian virus-40 (SV40) (7, 8), and by Herpes simplex virus (9), as well as for chemically induced tumors whose antigenic structure has not yet been clearly defined (10). The general consensus is that the MHC antigens involved in the recognition of the tumor target cell by the CTL are restricted to the antigens coded by two genes, *H-2K* and *H-2D*; the antigens coded by genes in the *H-2I* region and in other regions of *H-2* do not appear to be required for the recognition event (5, 11–13). Finally, the involvement of *H-2K* and *H-2D* has been shown not to be equivalent in all cases, such that tumor antigen recognition by CTL was preferential for an association of tumor antigen with *H-2K* or *H-2D* antigen (2, 5, 11–13), depending on the origin of the tumor and on the *H-2* haplotype of the CTL. Other studies have shown that this preferential recognition can be controlled by immune response (*Ir*) genes (11, 14, 15).

We recently reported (5) that recognition of a syngeneic Gross MuLV-induced tumor by CTL from BALB/c (*H-2^d*) mice appeared to involve the linked recognition of tumor antigen with *H-2K^d* antigen exclusively. However, efficient recognition of a syngeneic Gross MuLV-induced tumor by CTL from BALB.B (*H-2^b*) mice occurred in association with either *H-2K^b* or *H-2D^b* antigen (5, 16), in apparent contradiction to results reported by Green and colleagues (17) who showed that CTL from C57BL/6 (*H-2^b*) mice recognized a syngeneic Gross MuLV-induced tumor in preferential association with *H-2K^b*, not *H-2D^b* antigen.

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¹ Abbreviations used in this paper: CTL, cytolytic T lymphocyte; GCSA, Gross leukemia virus-induced cell surface antigen; *Ir*, immune response; MHC, major histocompatibility complex; MLTC, syngenic mixed leukocyte-tumor cell culture; SV40, Simian virus-40.

At least three possible explanations can be offered for this preference, or lack of preference, exhibited by CTL for a particular association of tumor antigen with *H-2K* or *H-2D* antigen. (a) The intensity of CTL-mediated lysis is strictly regulated by Ir genes, resulting in the more efficient recognition of certain tumor-*H-2* antigen combinations. (b) In the case of MuLV-induced tumors, the viral protein recognized by tumor-specific CTL may have different affinities for *H-2K*- or *H-2D*-coded molecules, and in consequence would be exposed on the cell surface mainly in association with one or the other of these two *H-2* antigens (12, 18). (c) The viral protein recognized by CTL does not possess a preferential binding affinity for *H-2K* or *H-2D* antigen; rather, there are quantitative differences in the amounts of *H-2K* or *H-2D* antigen exposed on the tumor cell surface due to metabolic or genetic control mechanisms at the tumor cell level (19).

The present communication describes comparative quantitative experiments designed to study the expression of *H-2K^d* and *H-2D^d* antigens by three different leukemia cell lines induced by Gross MuLV in BALB/c (*H-2^d*) mice. The preferential recognition of Gross MuLV-induced tumor antigen with *H-2K^d* or *H-2D^d* antigen by tumor-specific CTL from BALB/c mice was correlated with these quantitations. The three BALB/c tumor cell lines studied differed in the amounts of *H-2K^d* and *H-2D^d* antigens that they expressed on the cell surface, and these variations were reflected in their quality as target cells to Gross MuLV-specific CTL from BALB/c mice. Our results indicate that restriction of tumor-specific CTL activity by *H-2* can be directed at the target cell level by the quantitative modulation of *H-2K* or *H-2D* antigen expression, and suggest that separate tumors induced by the same MuLV in the same or in *H-2*-congenic strains of mice can vary in the absolute amounts of *H-2K* or *H-2D* antigen expressed on the cell surface.

Materials and Methods

Mice. All mice were bred at the Albert Einstein College of Medicine, Bronx, N. Y., or at Hôpital Cochin, Paris, from breeding pairs provided by Dr. F. Lilly. The inbred strains used included C57BL/6 (B6, *H-2^b*) and the *H-2*-congenic strains BALB/c (*H-2^d*), BALB.B (*H-2^b*), and BALB.G (*H-2^d*).

Tumor Cells. The following continuous leukemia cell lines induced by Gross MuLV in various congenic strains of mice were used: B.GV cells (*H-2^b*, *H-2K^bD^b*, BALB.B), C.GV cells (*H-2^d*, *H-2K^dD^d*, BALB/c); *H-2*-recombinant cells G.GV (*H-2^d*, *H-2K^dD^b*, BALB.G) and 5R.GV (*H-2^b*, *H-2K^bD^d*, BALB.5R); and F₁ hybrid B/CF₁.GV cells (*H-2^{b/d}*, [BALB.B × BALB/c]F₁). The origin and characteristics of these cell lines have been described previously (5). In addition, two new cell lines designated "C.GV-300" and "C.GV-302" were induced in BALB/c mice by infection with Gross MuLV and were established as continuous suspension cultures (see Results), according to the method described by Freedman and Lilly (20). All cell lines were maintained as stationary suspension cultures in Eagle's minimal essential medium supplemented with 10% fetal bovine serum.

Antisera. The following mouse antisera were prepared in our laboratories by repeated immunization: anti-*H-2^b* serum ([BALB/c × C₃H]F₁ anti-EL4 [*H-2^b*] leukemia cells); anti-*H-2D^b* (H-2.2) serum (BALB/c anti-BALB.G spleen cells); anti-*H-2K^b* (H-2.33) serum ([A × BALB.G]F₁ anti-BALB.B spleen cells); anti-*H-2^d* serum ([C57BL/6 × C57BL/10.BR]F₁ anti-Meth-A [*H-2^d*] leukemia cells); anti-*H-2K^d* (H-2.31) serum (BALB.B anti-BALB.G spleen cells; absorbed twice on one-half volumes of packed B10.A spleen cells); anti-*H-2D^d* (H-2.4) serum (BALB.G anti-BALB/c spleen cells); anti-Gross leukemia virus-induced cell surface antigen (GCSA) serum ([BALB.B × BALB/c]F₁ anti-B/CF₁.GV leukemia cells). Goat antiserum to Rauscher MuLV gp69/71 antigen was obtained through the Office of Program Resources and

Logistics, Viral Oncology Division, National Cancer Institute, National Institutes of Health, Bethesda, Md. All antisera were heat inactivated at 56°C for 30 min.

Lymphocyte Cultures. Gross MuLV-specific CTL were generated in syngeneic secondary mixed leukocyte-tumor cell cultures (MLTC) from spleen cells of primed mice, as described elsewhere (5). CTL specific for *H-2* alloantigens were generated in primary mixed leukocyte cultures (16, 21).

Lymphocyte Clones. CTL clones were derived from lymphocytes harvested in MLTC and distributed in multiwell plates at limiting dilutions ranging from 0.5 to 1.0 cell/well in the presence of 30% conditioned medium and 1×10^6 x-irradiated (3,000 rad) syngeneic spleen cells. Cloning efficiency ranged from 64 to 77%. Conditioned medium came from rat spleen cells incubated at 37°C for 36 h with 5 µg/ml concanavalin A. Each CTL culture was fed every 4-5 d with conditioned medium; after 4 wk each clone was transferred into upright tissue culture flasks and expanded by the addition of 30% conditioned medium, 3×10^6 x-irradiated (3,000 rad) syngeneic spleen cells, and 1×10^6 x-irradiated (5,000 rad) tumor cells as a source of antigen.

⁵¹Cr Release Cytotoxicity Assays. Cell-mediated antitumor cytolytic activity was detected using a modification (5) of the method of Brunner et al. (22). All assays were terminated after 6 h incubation at 37°C. Spontaneous release values varied between 4 and 15% of the total incorporated label. Antibody- and complement-mediated cytotoxicity was detected with the method originally described by Sanderson (23) using 2.5×10^4 ⁵¹Cr-labeled target cells and agarose-adsorbed rabbit complement (24) in 45-min assays.

Inhibition Assays. CTL specificity for target antigens was analyzed by two approaches: (a) addition of varying numbers of competitor target cells to the ⁵¹Cr release cytotoxicity assay (5) and (b) addition of anti-*H-2* sera at varying dilutions, in the absence of complement, to the ⁵¹Cr release cytotoxicity assay (1, 11). The percentage of inhibition of CTL-mediated cytotoxicity by both approaches was calculated according to the formula: control - experiment/control × 100, where control was the specific cytotoxic activity detected in positive control wells; and experiment was the experimental cytotoxicity values obtained from wells containing competitor target cells or blocking antisera.

Quantitative Absorption of Specific Antisera. All sera to be absorbed were used at dilutions giving 40-50% lysis of a standard target cell following incubation in the presence of rabbit complement. Cell surface antigens were quantitated by incubating 100 µl of specific antisera with varying numbers of normal spleen cells or tumor cells for 30 min at 0°C. The cells were then removed by centrifugation and the remaining cytotoxic activity of the antiserum was assayed on ⁵¹Cr-labeled target cells in the presence of rabbit complement (see above), and was compared to the activity of sham-absorbed antiserum at the same dilutions. The percentage of absorption of specific antiserum activity was calculated with the formula given above.

Results

Establishment and Initial Characterization of New BALB/c Gross MuLV-induced Tumor Cell Lines. Six BALB/c mice from the same litter were inoculated with 0.05 ml of a cell-free extract of Gross MuLV (25) on the first day following birth. Four mice developed leukemia 3-5 mo later, and upon inspection had hyperplastic thymus, spleen, liver, and/or mesenteric lymph nodes. The leukemic lymphoid organs were removed, minced separately and inoculated subcutaneously into adult BALB/c mice of the same sex as the leukemic mouse, according to the protocol established by Freedman and Lilly (20). Following the appearance of solid tumors, the leukemic cells were extracted and established as suspension cultures. After 10 passages in vitro each tumor cell line was checked for its ability to incorporate ⁵¹Cr and for its quality as a target to Gross MuLV-specific CTL. Two particular cell lines, C.GV-300 and C.GV-302, were selected for extensive study. These two lines originated from the same leukemic thymus but were passaged as subcutaneous tumors in two different adult BALB/c mice (numbers 300 and 302), and subsequently were maintained as independent

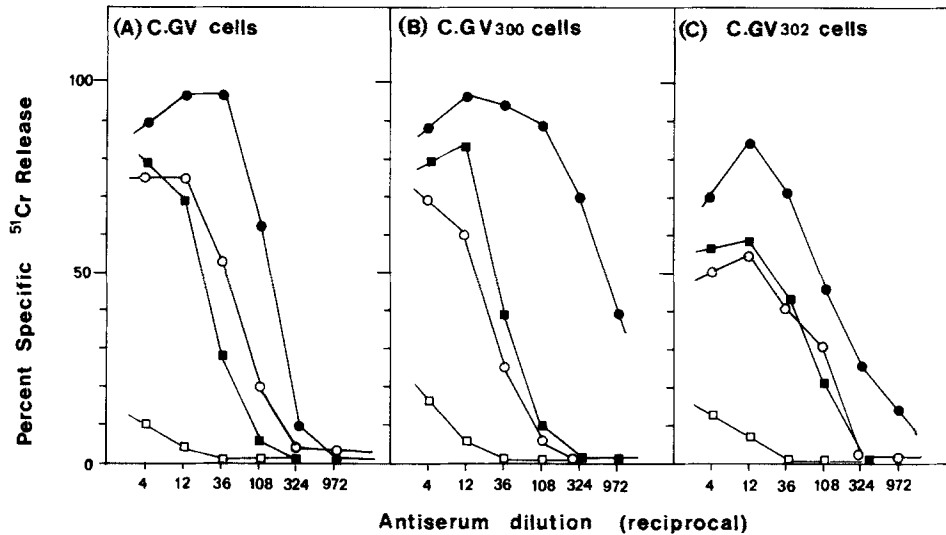


FIG. 1. Detection of *H-2K^d* and *H-2D^d* antigens on the surface of (A) C.GV cells, (B) C.GV-300 cells, and (C) C.GV-302 cells by the incubation of ⁵¹Cr-labeled tumor cells in the presence of anti-*H-2* serum and rabbit complement. ●, anti-*H-2^d* serum; ○, anti-*H-2A* (*H-2D^d*) serum; ■, anti-*H-2.31* (*H-2K^d*) serum; and □, normal mouse serum.

suspension cultures. Preliminary experiments revealed that these two "sister" lines differed with respect to their qualities as targets to syngeneic Gross MuLV-specific CTL, C.GV-300 cells being more susceptible to lysis than C.GV-302.

Cell-Surface Antigen Expression by Gross MuLV-induced Tumor Cell Lines. In view of the differences in their qualities as target cells to Gross MuLV-specific CTL, lines C.GV-300 and C.GV-302 were analyzed serologically with respect to their principal surface antigens in comparison with the C.GV line established previously (5, 16). Detection of the GCSA (26) and of the MuLV glycoprotein gp70 by quantitative absorption of specific antisera showed that the three cell lines were positive in comparable degrees for both antigens (data not shown).

In contrast, analysis of the expression of *H-2* antigens revealed major differences among the three cell lines. Fig. 1 indicates that all three lines expressed a sufficient number of surface molecules of *H-2A* (*H-2D^d*) and *H-2.31* (*H-2K^d*) antigens for lysis to occur following incubation in the presence of monospecific anti-*H-2* sera and rabbit complement. However, as shown in Fig. 2, quantitative absorption of these antisera with varying numbers of normal BALB/c spleen cells or with C.GV, C.GV-300, or C.GV-302 cells indicated that C.GV cells expressed barely detectable levels of *H-2A* (*H-2D^d*) antigen, whereas C.GV-300 cells expressed relatively large amounts of the same antigen (Fig. 2 A). C.GV-302 cells were intermediate, since they expressed 7.5 times less *H-2A* antigen than C.GV-300 cells and at least 10 times more *H-2A* antigen than C.GV cells. Quantitation of the amount of *H-2.31* (*H-2K^d*) antigen expressed on the three cell lines (Fig. 2 B) showed that the C.GV and C.GV-300 lines were roughly equivalent and that the C.GV-302 cell line expressed three times less *H-2.31* antigen than the C.GV or the C.GV-302 line. The apparent scarcity of *H-2.31* antigen on these tumor cells relative to normal BALB/c spleen cells could be due to the presence of contaminating anti-Ia antibodies in the anti-*H-2.31* serum in spite of absorption of

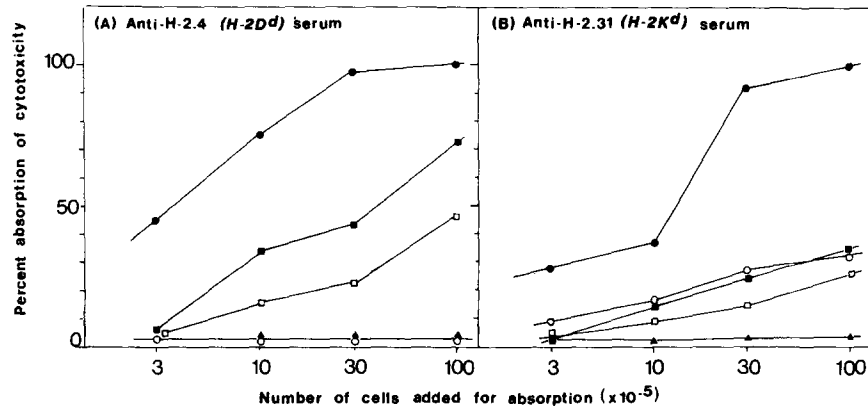


FIG. 2. Quantitation of $H-2D^d$ and $H-2K^d$ antigen expression following absorption of (A) anti-H-2.4 ($H-2D^d$) serum and (B) anti-H-2.31 ($H-2K^d$) serum by BALB/c spleen cells (●), C.GV cells (○), C.GV-300 cells (■), C.GV-302 cells (□), or BALB.B spleen cells (▲). Cytotoxicity was determined by incubation of ^{51}Cr -labeled BALB/c spleen cells in the presence of the absorbed sera and rabbit complement. Unabsorbed sera gave the following degrees of cytotoxicity in the presence of rabbit complement: anti-H-2.4 serum (1/50), 54%; anti-H-2.31 serum (1/100), 40%; normal mouse serum (1/50), 4%.

the antiserum on B10.A spleen cells (see Materials and Methods). Since spleen cells are rich in Ia antigens, as opposed to the tumor cells in question, an exaggerated difference in the binding of anti-H-2.31 serum antibodies would be observed. This problem will be resolved by quantitation of H-2.31 antigen using monoclonal anti-H-2.31 antibodies. The present results thus indicated that the three tumor cell lines studied varied widely in the amounts of $H-2$ antigens that they expressed, C.GV-300 cells expressing relatively high levels of both H-2.4 and H-2.31 antigens, C.GV-302 cells being intermediate, and C.GV cells expressing high levels of H-2.31 antigen but barely detectable levels of H-2.4 antigen.

A further confirmation of the disparity in $H-2K^d$ and $H-2D^d$ antigen expression among our tumor cell lines came from the analysis with $H-2$ -specific CTL. Table I shows that all three tumor cell lines were susceptible to lysis by BALB.B anti-BALB/c (anti- $H-2^d$) CTL. Analysis with monospecific CTL showed that C.GV-300 cells were killed by both anti- $H-2K^d$ and anti- $H-2D^d$ CTL. C.GV-302 cells were also killed by both types of CTL, but 3–10 times less efficiently. Finally, C.GV cells were only killed by anti- $H-2K^d$ CTL. The degree of specificity of these CTL was indicated by the fact that none killed B.GV ($H-2^b$) cells efficiently, that anti- $H-2K^d$ CTL killed $H-2$ -recombinant G.GV ($H-2K^dD^b$) cells and not 5R.GV ($H-2K^bD^d$) cells, and that anti- $H-2D^d$ CTL killed 5R.GV cells and not G.GV cells. The data shown in Table I thus indicated that the expression of relatively large amounts of $H-2$ antigen was required on the surface of the target cells in order for efficient CTL-mediated lysis to occur. Moreover, efficient complement-mediated lysis in the presence of specific antibody required the expression of much smaller amounts of $H-2$ antigen, the expression of a few $H-2$ molecules on the cell surface probably being sufficient for antibody binding to occur and for lethal membrane damage to be initiated (cf. Fig. 1).

Effect of H-2 Antigen Expression on CTL Generation and Activity. Previously, we reported that the BALB/c C.GV cell line had the capacity of inducing large amounts of Gross

TABLE I
Detection of H-2K^d and H-2D^d Antigens on C.GV Tumor Cells Using H-2-specific CTL

Designation	Target cells		Percentage of specific ⁵¹ Cr release											
	<i>H-2</i> Genotype		BALB.B Anti-BALB/c (anti- <i>H-2K^dD^d</i>)				BALB.B Anti-BALB.G (anti- <i>H-2K^d</i>)				BALB.G Anti-BALB/c (anti- <i>H-2D^d</i>)			
	K	D	100	30	10	3	100	30	10	3	100	30	10	3
C.GV	d	d	19	17	12	0	21	17	2	0	0	0	0	0
C.GV-300	d	d	62	59	43	13	56	54	20	9	46	38	15	2
C.GV-302	d	d	25	21	5	1	27	24	5	0	18	6	3	0
G.GV	d	b	34	32	12	5	48	44	31	14	0	0	0	0
5R.GV	b	d	40	30	22	3	14	8	0	0	37	21	10	1
B.GV	b	b	7	2	3	0	7	4	3	1	11	4	1	0

CTL were harvested after 6d incubation in MLC, and tested for cytotoxicity on a panel of ⁵¹Cr-labeled target cells at the indicated lymphocyte to target cell ratios in a 6-h assay.

TABLE II
H-2 Specificity of GV-specific CTL from BALB/c and BALB.B Mice

Designation	Target cells		Percentage of specific ⁵¹ Cr release															
	<i>H-2</i> Genotype		BALB/c Anti-C.GV				BALB/c Anti-C.GV-300				BALB/c Anti-C.GV-302				BALB.B Anti-B.GV			
	K	D	30:1	10:1	3:1	1:1	30:1	10:1	3:1	1:1	30:1	10:1	3:1	1:1	30:1	10:1	3:1	1:1
C.GV	d	d	6	4	0	0	11	2	0	0	2	1	0	0	0	0	0	0
C.GV-300	d	d	43	25	12	6	66	47	30	13	33	15	15	2	7	3	0	0
C.GV-302	d	d	35	22	17	4	54	22	9	7	29	17	9	4	6	4	2	0
B/C F ₁ GV	b/d	b/d	16	8	3	1	28	11	5	2	18	12	9	1	12	4	1	0
G.GV	d	b	43	26	16	6	55	32	14	11	19	12	10	5	26	14	10	2
5R.GV	b	d	0	1	0	0	36	18	7	2	9	5	2	0	16	9	4	0
B.GV	b	b	0	0	0	0	2	1	1	0	2	0	3	2	66	42	27	10

Lymphocytes were harvested after 6d incubation in secondary MLTC, and tested against a panel of ⁵¹Cr-labeled target cells at the indicated lymphocyte to target cell ratios in a 6-h assay.

MuLV-specific CTL in secondary MLTC, but that C.GV cells themselves were very poor targets to the CTL they induced (5). Furthermore, the Gross MuLV-specific CTL generated were restricted to Gross MuLV-induced cells that expressed *H-2K^d* antigen; *H-2D^d* antigen appeared to be irrelevant for CTL recognition of the target cell to occur (5). We have consistently reproduced this paradoxical situation: Table II shows that BALB/c (*H-2^d*) CTL induced by C.GV cells were incapable of killing C.GV target cells, but killed C.GV-300 and C.GV-302 cells efficiently. B/CF₁GV (*H-2^{b/d}*) cells, induced by Gross MuLV in (BALB.B × BALB/c)F₁ hybrid mice, were also susceptible to lysis by C.GV-induced CTL, as were G.GV (*H-2K^dD^b*) tumor cells. As reported previously (5), 5R.GV (*H-2K^bD^d*) and B.GV (*H-2^b*) cells were insensitive to lysis mediated by C.GV-induced CTL.

Attempts to generate CTL in BALB/c mice against syngeneic C.GV-300 or C.GV-302 tumor cells showed that these two cell lines, in contrast to C.GV cells, were capable both of inducing Gross MuLV-specific CTL in MLTC and of serving as targets to the lytic activity of these CTL. Table II shows that C.GV-300 cells were both better stimulators of CTL differentiation in MLTC and better targets to CTL

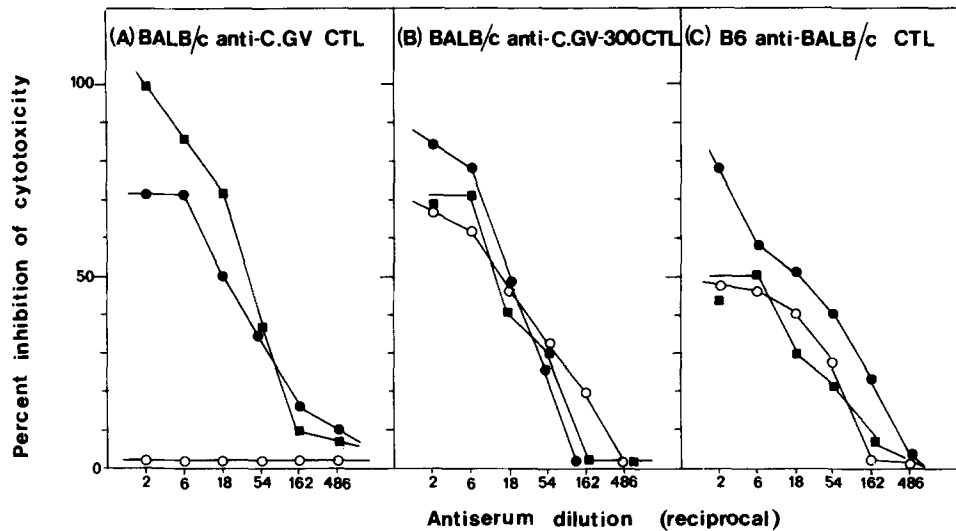


FIG. 3. Inhibition of CTL-specific activities by incubation of ^{51}Cr -labeled C.GV-300 ($H-2^d$) target cells with anti- $H-2$ sera in the absence of complement. CTL were assayed at a ratio of 10 lymphocytes to 1 target cell. Cytotoxicity values in the presence of normal mouse serum (1/2-1/486) were: (A) BALB/c anti-C.GV CTL, 31%; (B) BALB/c anti-C.GV-300 CTL, 25%; (C) B6 anti-BALB/c CTL, 17%. ●, anti- $H-2^d$ serum; ○, anti- $H-2.4$ ($H-2D^d$) serum; ■, anti- $H-2.31$ ($H-2K^d$) serum.

lytic activity than either C.GV-302 or C.GV cells. Table II also demonstrates that C.GV-300 cells were capable of inducing Gross MuLV-specific CTL capable of recognizing tumor antigen in association with either $H-2K^d$ antigen (i.e., G.GV target cells) or $H-2D^d$ antigen (i.e., 5R.GV target cells). C.GV-302 cells induced CTL restricted mainly to $H-2K^d$, although detectable lytic activity was also directed against $H-2D^d$ antigen. That these CTL were specific for the $H-2^d$ haplotype was indicated by the fact that they killed C.GV-300 and C.GV-302 ($H-2^d$) cells, as well as B/CF₁.GV ($H-2^{b/d}$) cells, but not B.GV ($H-2^b$) cells. The latter, however, were killed by B.GV-immune CTL from BALB.B mice (Table II), which could recognize tumor antigen in association with either $H-2K^b$ antigen (i.e., 5R.GV cells) or $H-2D^b$ antigen (i.e., G.GV cells). The results summarized in Table II thus showed that Gross MuLV-specific CTL recognizing $H-2K$ and $H-2D$ antigens could be generated in MLTC with immune spleen cells from either BALB/c or BALB.B mice; and that, in the case of BALB/c mice, the presence of $H-2D^d$ -specific CTL depended on the choice of the Gross MuLV-induced tumor cells used as stimulators in MLTC.

That C.GV-300 tumor cells are capable of inducing Gross MuLV-specific CTL in BALB/c mice specific for either $H-2K^d$ or $H-2D^d$ antigen was confirmed by three independent approaches:

(a) *Inhibition of CTL-specific activity by anti- $H-2$ sera in the absence of complement:* Fig. 3 summarizes data concerning the inhibition by antibodies of Gross MuLV-specific CTL generated in MLTC from BALB/c immune spleen cells. C.GV-300 tumor cells were chosen as target cells throughout since they express optimal amounts of both $H-2K^d$ and $H-2D^d$ antigens. Stimulation in MLTC with C.GV tumor cells (Fig. 3A) resulted in CTL that could be blocked by incubation of the ^{51}Cr -labeled target cells with anti- $H-2^d$ serum (containing antibodies against both $H-2K^d$ and $H-2D^d$ antigens) and with anti- $H-2.31$ ($H-2K^d$) serum. Anti- $H-2.4$ (anti- $H-2D^d$) serum failed to block

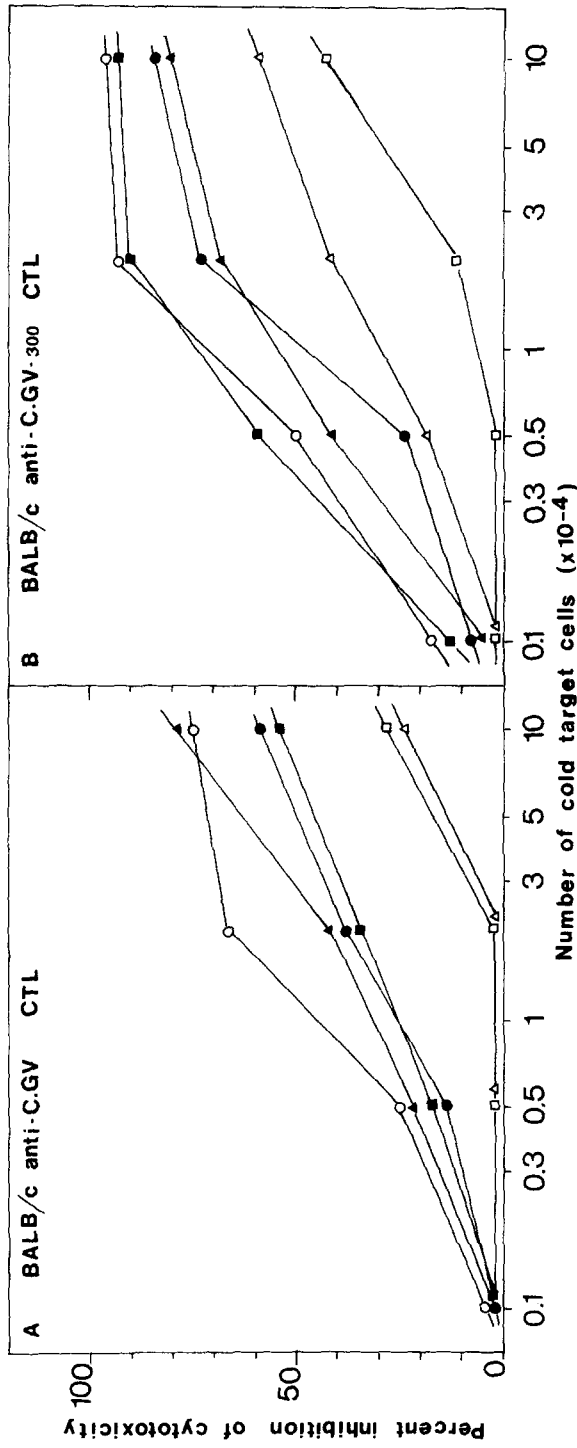


Fig. 4. Competitive inhibition of CTL activities with unlabeled target cells. BALB/c anti-C.GV CTL (A) and BALB/c anti-C.GV-300 CTL (B) were assayed on ^{51}Cr -labeled C.GV-300 target cells at a ratio of 30:1 in a 6-h assay, and gave cytotoxicity values of 33 and 46%, respectively. Increasing numbers of unlabeled tumor cells were added to each cell mixture, as follows: ●, C.GV cells; ○, C.GV-300 cells; ■, C.GV-302 cells; □, C.GV cells; ▲, B.GV cells; △, 5R.GV cells.

TABLE III
H-2 Specificity of Cloned GV-immune CTL from BALB/c Mice

Clone group	Cytotoxic activity detected on			Frequency*	
	C.GV-300 (<i>H-2K^dD^d</i>)	G.GV (<i>H-2K^dD^b</i>)	5R.GV (<i>H-2K^bD^d</i>)	Anti-C.GV	Anti-C.GV-300
I	+	+	-	0.43 (18/42)	0.45 (9/20)
II	+	-	+	0.12 (5/42)	0.35 (7/20)
III	+	+	+	0.33 (14/42)	0.20 (4/20)

CTL clones were grown by limiting dilution from BALB/c anti-C.GV or BALB/c anti-C.GV-300 lymphocytes. CTL were generated in secondary MLTC and distributed in multiwell plates at a concentration of 0.5 cell/well. Starting on day 4 after distribution, the wells were inspected microscopically every day, and those cultures which presented single proliferating cell clusters were defined as lymphocyte clones. Following expansion, each clone was tested for cytotoxicity three different times on a panel of ⁵¹Cr-labeled target cells in 6-h assays.

* Number of clones positive/total number of clones assayed.

CTL activity, indicating the absence of Gross MuLV-specific CTL which recognized tumor antigen in association with *H-2D^d* antigen.

Fig. 3B indicates that C.GV-300 tumor cells generated two independent subsets of Gross MuLV-specific CTL, one subset recognizing *H-2K^d* and the other *H-2D^d* antigen, because both anti-H-2.31 and anti-H-2.4 sera blocked CTL-mediated lysis efficiently. It should be noted that neither of the two monospecific antisera could inhibit killing completely at any of the dilutions assayed, thus suggesting the independent activity of two subsets of CTL. An analogous finding was observed with alloantigen-specific CTL (Fig. 3C) generated by stimulating C57BL/6 ("B6," *H-2^b*) spleen cells with x-irradiated normal BALB/c (*H-2^d*) spleen cells in MLC: one CTL subset (i.e., anti-*H-2K^d* CTL) was inhibited by anti-H-2.31 serum, whereas a different subset appeared to be inhibited by anti-H-2.4 serum, neither serum giving complete inhibition of CTL activity.

(b) *Competitive inhibition of CTL specific activity by unlabeled tumor cells*: Fig. 4A shows that BALB/c anti-C.GV CTL, when tested on ⁵¹Cr-labeled C.GV-300 target cells, can be inhibited efficiently by the addition of unlabeled C.GV, C.GV-300, C.GV-302, and G.GV (*H-2K^dD^b*) cells; 5R.GV (*H-2K^bD^d*) and B.GV (*H-2^b*) cells were poor inhibitors of cytotoxicity, thus indicating that BALB/c anti-C.GV CTL were restricted to *H-2K^d* antigen. On the other hand, Fig. 4B shows that BALB/c anti-C.GV-300 CTL were inhibited by both G.GV and 5R.GV cells, as well as by C.GV, C.GV-300, and C.GV-302 cells. C.GV-300 cells, which express appreciable amounts of both *H-2K^d* and *H-2D^d* antigens, were better inhibitors of CTL activity than either G.GV or 5R.GV cells, indicating the existence of *H-2K^d*- and *H-2D^d*-restricted CTL subsets.

(c) *Specificity analysis of cloned CTL*: Table III summarizes the results obtained after cloning BALB/c anti-C.GV CTL and BALB/c anti-C.GV-300 CTL. CTL clones were obtained by limiting dilution of CTL from MLTC and expansion in the presence of conditioned medium, x-irradiated BALB/c spleen feeder cells, and x-irradiated C.GV or C.GV-300 tumor cells as a source of antigen. When assayed for cytotoxicity on a panel of ⁵¹Cr-labeled tumor cells, the CTL could be grouped into three categories. Group I contained the largest number of clones (43–45%) and included the cloned CTL that lysed both C.GV-300 and G.GV (*H-2K^dD^b*) target cells, but not 5R.GV (*H-2K^bD^d*) cells; this pattern of specificity indicated that the CTL clones in group I

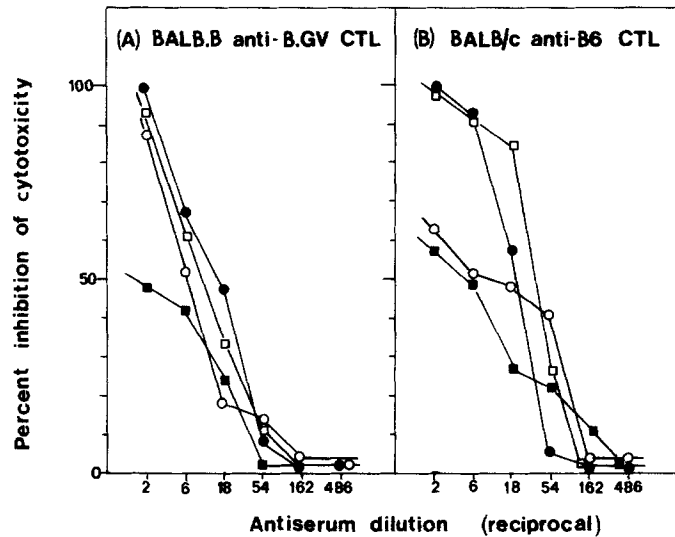


FIG. 5. Inhibition of CTL-specific activities by incubation of ^{51}Cr -labeled B.GV ($H-2^b$) target cells with anti- $H-2$ sera in the absence of complement. BALB.B anti-B.GV CTL (A) and BALB/c anti-B6 CTL (B) were tested in a 6-h assay at a ratio of 10 lymphocytes to 1 target cell, and gave cytotoxicity values of 33 and 41%, respectively, in the presence of normal mouse serum (1/3-1/243). ●, anti- $H-2^b$ serum; ○, anti- $H-2.2$ ($H-2D^b$) serum; ■, anti- $H-2.33$ ($H-2K^b$) serum; □, anti- $H-2.2$ + anti- $H-2.33$ sera (1:1).

were restricted to $H-2K^d$ antigen. Group II included CTL clones that recognized and killed C.GV-300 and 5R.GV cells, but not G.GV cells, and consequently were restricted to $H-2D^d$ antigen. Only 12% (5/42) of the cloned BALB/c anti-C.GV CTL fell in this category; however, a significantly higher proportion (i.e., 35% or 7/20) of CTL clones fell in this category when BALB/c anti-C.GV-300 CTL were considered. Group III included CTL clones that killed C.GV-300, G.GV, and 5R.GV target cells; this group included 20-33% of all clones. Extended analysis showed that CTL clones in group III also killed an assortment of "irrelevant" target cells, indicating the lack of an astringent degree of specificity for the immunizing tumor cell.² These results consequently showed that BALB/c anti-C.GV CTL and BALB/c anti-C.GV-300 CTL consisted of at least three subsets of cytolytic T cells, two subsets recognizing either $H-2K^d$ or $H-2D^d$ antigen independently, and a third subset recognizing both antigens. Furthermore, the choice of the tumor cell used to generate CTL appeared to determine the relative proportion of each CTL subset. A detailed description of these CTL clones is provided in a separate communication.²

Analysis of BALB.B anti-B.GV CTL Specificities. In a previous report (5) we indicated that CTL from BALB.B ($H-2^b$) mice immunized against syngeneic Gross MuLV-induced B.GV cells recognized tumor antigen on B.GV cells in association with either $H-2K^b$ or $H-2D^b$ antigen. This conclusion was drawn from studies concerning the direct lysis of G.GV ($H-2K^dD^b$) and 5R.GV ($H-2K^bD^d$) target cells by BALB.B CTL. A new series of experiments confirmed the latter observations. Fig. 5A shows that CTL-specific activity directed against ^{51}Cr -labeled B.GV target cells was inhibited by anti- $H-2^b$ serum, as well as by anti- $H-2.2$ (anti- $H-2D^b$) and anti- $H-2.33$ (anti- $H-2K^b$)

² Plata, F. Specificity studies of cytolytic T lymphocytes (CTL) directed against murine leukemia virus-induced tumors. Analysis by monoclonal CTL. Manuscript submitted for publication.

TABLE IV
H-2 Specificity of CTL Clones Generated from BALB.B Anti-B.GV Lymphocytes

Clone group	Cytotoxic activity detected on			Frequency*
	B.GV (<i>H-2K^bD^b</i>)	G.GV (<i>H-2K^dD^b</i>)	5R.GV (<i>H-2K^bD^d</i>)	
I	+	+	–	0.48 (12/25)
II	+	–	+	0.16 (4/25)
III	+	+	+	0.36 (9/25)

CTL clones were grown by limiting dilution at 1.0 cell/microplate well from BALB.B anti-B.GV lymphocytes harvested from secondary MLTC. CTL clones were defined as described for Table III. Each CTL clone was tested for cytotoxicity four independent times on a panel of ⁵¹Cr-labeled target cells in 6-h assays.

* Number of clones positive/total number of clones assayed.

sera. Moreover, a 1:1 mixture of anti-H-2.2 and anti-H-2.33 sera inhibited CTL activity more efficiently than either serum alone. These results thus indicated that Gross MuLV-induced tumor antigens could be recognized in association with either *H-2K^b* or *H-2D^b* antigens, and that each "association" was probably recognized by a different subset of Gross MuLV-specific CTL. A control assay with alloantigen-specific BALB/c anti-B6 (anti-*H-2^b*) CTL assayed on B.GV target cells (Fig. 5B) also indicated the existence of two independent subsets of CTL, one recognizing *H-2K^b* antigen and the other *H-2D^b*.

The specificity analysis of 25 BALB.B anti-B.GV CTL clones confirmed the results above. Table IV shows that the majority of these CTL clones (i.e., 48% or 12/25, group I) recognized and killed B.GV cells and G.GV (*H-2K^dD^b*) cells, but not 5R.GV (*H-2K^bD^d*) cells. The clones in group I thus appeared to recognize Gross MuLV-induced antigen in association with *H-2K^b* antigen, and not with *H-2D^b* antigen. Group II (four clones) gave the inverse specificity pattern. Finally, group III (nine clones) seemed to recognize tumor antigen in association with both *H-2D^b* and *H-2K^b* antigens, and lacked a high degree of specificity, as indicated by extended analysis.² These results were analogous to the specificity patterns observed with BALB/c anti-C.GV-300 CTL clones, where at least three different categories of CTL clones were also identified.

Discussion

In this study we have considered by quantitative approaches the expression of *H-2K* and *H-2D* antigens on the surface of three different BALB/c tumor cell lines induced by Gross MuLV. Quantitative absorption of anti-H-2.31 serum with cells from these three lines indicated that C.GV and C.GV-300 cells expressed roughly equivalent amounts of *H-2K^d* antigen, whereas C.GV-302 cells expressed three times less *H-2K^d* antigen than cells from either one of the other two lines (Fig. 2). Moreover, expression of *H-2D^d* antigen varied widely from one tumor cell line to the other, thus, C.GV cells expressed barely detectable amounts, G.GV-300 cells expressed relatively large amounts of *H-2D^d* antigen, and C.GV-302 cells expressed intermediate levels of *H-2D^d* antigen (7.5 times less than the equivalent number of C.GV-300 cells). An interesting observation was that in all cases normal BALB/c spleen cells expressed more (i.e., 7.5–100 times) *H-2* antigens than any of the BALB/c tumor cell lines

studied. Furthermore, it should be stressed that the C.GV-300 and C.GV-302 cell lines were derived from the same original Gross MuLV-induced thymoma, and divergence with respect to *H-2D^d* antigen expression must have occurred during the course of establishment of the two tumor cell lines as continuous suspension cultures. In this context, Scollay et al. (27) have shown that thymocyte subpopulations can vary significantly in the quantitative expression of *H-2* antigens. Likewise, we recently reported that MuLV-induced tumor cells can show wide phenotypic variations when considered at the clonal level (21). In view of these facts, it is not surprising that two variant cell lines originating from the same neoplasm were selected during the course of their establishment as continuous tumor cell lines in vitro. An interesting corollary was that expression of Gross MuLV-induced cell surface antigens (i.e., GCSA and gp70), as detected by specific antisera, were not significantly altered by the depressed expression of *H-2D^d* antigen in any one of the cell lines studied.

Analysis of the three cell lines with anti-*H-2* CTL yielded results comparable to those obtained by serological analysis: all three lines displayed detectable amounts of *H-2K^d* antigen, but varied quantitatively in the expression of *H-2D^d* antigen (Table I). Thus, *H-2D^d*-specific CTL were not capable of killing C.GV target cells, but killed C.GV-300 and C.GV-302 cells. It was apparent that, of all the target cells assayed, C.GV-300 cells were the most sensitive to lysis mediated by anti-*H-2* CTL, possibly due to the expression of optimal amounts of *H-2K^d* and *H-2D^d* antigens by C.GV-300 cells.

The differences in the expression of *H-2D^d* antigen by C.GV, C.GV-300, and C.GV-302 tumor cells affected the *H-2* restriction patterns of the Gross MuLV-specific CTL which these tumor cells elicited in MLTC, as shown concordantly by experiments involving three different technical approaches: (a) inhibition of CTL activity by monospecific anti-*H-2* sera; (b) competitive inhibition of CTL-mediated cytotoxicity by the addition of excess tumor cells into the reaction mixture; and (c) clonal analysis of CTL specificities. All three approaches showed that C.GV-300 tumor cells induced both *H-2K^d*- and *H-2D^d*-specific CTL in MLTC, while C.GV tumor cells mainly induced *H-2K^d*-specific CTL. An intermediate situation was observed with C.GV-302 cells, since they induced both *H-2K^d*- and *H-2D^d*-specific CTL; *H-2K^d*-specific CTL were clearly in excess (Table II).

A similar analysis of Gross MuLV-specific CTL from BALB.B (*H-2^b*) mice indicated that syngeneic B.GV tumor cells induced the generation of CTL that recognized tumor antigen in association with *H-2K^b* and *H-2D^b* antigens, with a possible excess of *H-2K^b*-specific CTL, confirming our previous observations (5, 16). In apparent contradiction, Green and collaborators (17) indicated that Gross MuLV-specific CTL from C57BL/6 (*H-2^b*) mice recognized Gross MuLV-induced antigens on E δ G2 (*H-2^b*) leukemia cells in association with *H-2K^b* antigen, and not with *H-2D^b* antigen. Extrapolation of our results concerning the three tumor cell lines induced by Gross MuLV in BALB/c (*H-2^d*) mice to the *H-2^b* system leads to the suggestion that B.GV cells might express higher quantities of *H-2D^b* antigen than E δ G2 cells, and that the proportions of *H-2K^b*- and *H-2D^b*-specific CTL generated by each tumor cell vary accordingly. A direct comparison of these two systems of immunity directed against Gross MuLV antigens in *H-2^b* mice remains to be performed.

Another unresolved problem is the intriguing dissociation exhibited by tumor cells with respect to their qualities as stimulator cells for CTL generation in syngeneic

MLTC, as opposed to their qualities as target cells to tumor-specific CTL in the ^{51}Cr release assay (5, 12). Both C.GV-300 and C.GV-302 leukemia cells were good stimulators of CTL generation and were adequate target cells to CTL-mediated lysis, although C.GV-300 cells proved to be consistently better than C.GV-302 cells with respect to both functions. In contrast, as reported previously (5), C.GV cells were excellent stimulators of CTL differentiation in syngeneic MLTC but were very poor targets to Gross MuLV-specific CTL-mediated lysis. The minimal expression of $H-2D^d$ antigen by C.GV cells cannot explain this dissociation completely, since C.GV cells were adequate targets to alloantigen-specific CTL directed against $H-2K^d$ alloantigen (Table I). Furthermore, C.GV cells express Gross MuLV-induced antigens, as determined by serological techniques, and can compete for the lysis of ^{51}Cr -labeled C.GV-300 target cells (Fig. 4).

Our experiments suggest that the pattern of $H-2$ restriction observed among tumor-specific CTL might occasionally be directed by the quantitative modulation of $H-2K$ or $H-2D$ antigen expression by the tumor cell. The selective shut-down of cell surface antigen expression by eucaryotic cells is a phenomenon that has been extensively studied in models ranging from protozoa (28, 29) to mammalian lymphocytes and tumor cells (30–33). The existence of a primitive (from the evolutionary point of view) reaction leading to the suppression of individual cell surface antigens has been established. The mechanism underlying the selective repression of $H-2$ antigens is not yet understood; hypotheses have been proposed, ranging from the selection of cells having suffered structural mutation in $H-2$ to the selection of cells in which portions of chromosome 17 (which contains the MHC) have been selectively inactivated (32, 33). In this context, Jones and Bodmer (34) have reported the identification of human tumor cells deficient in the expression of cell surface HLA transplantation antigens identifiable by specific antibody, suggesting that the repression of a single gene in tumor cells might facilitate escape from the selective pressure of immune surveillance.

Normal lymphocytes can also show variations in the expression of $H-2$ antigens; Emerson et al. (19) reported data indicating that $H-2K$, $H-2D$, and $H-2I$ antigens are spontaneously shed at different rates from viable normal lymphocytes in culture, in the absence of antibody. Furthermore, the rate of shedding of individual antigens varied among lymphocytes from different strains of mice, and this rate was controlled by genes mapped to the MHC (19). Because replacement of shed $H-2$ antigens on the cell surface is a relatively slow process, rapid shedding of an individual $H-2$ antigen and variations in the rate of replacement could lead to quantitative differences in the expression of the antigen at the cellular level.

If this analysis is applicable to tumor cells, $H-2$ restriction patterns of tumor cell recognition by tumor-specific CTL would be affected. A report by Gooding (12) recently indicated that SV40-transformed ($H-2^k \times H-2^b$) F_1 hybrid cells expressed suboptimal amounts of the $H-2K^b$ /SV40 target antigen recognized by specific CTL. The same cells, however, expressed adequate amounts of SV40 antigen associated with $H-2K^k$, $H-2D^b$, and $H-2D^k$ antigens. Although Gooding explained these results by invoking an increased avidity of SV40 antigen for $H-2K^k$ relative to $H-2K^b$ antigen, a decrease in the amount of $H-2K^b$ antigen on the cell surface would also provide a plausible explanation.

The mechanism responsible for the variability in $H-2$ antigen expression by our tumor cell lines induced by Gross MuLV in BALB/c mice is not understood. It

remains to be specified whether this variability is the result of differential rates in the shedding of *H-2K* and *H-2D* antigens, or whether, on the contrary, it is the result of an activation phenomenon of *H-2* genes, such as was reported by Meruelo (35) concerning the heightened expression of *H-2* antigens on tumor cells after incubation in the presence of interferon.

Summary

Comparative quantitative experiments were designed to study the expression of *H-2K^d* and *H-2D^d* antigens on three different leukemia cell lines induced by Gross murine leukemia virus (MuLV) in BALB/c (*H-2^d*) mice. The *H-2* restriction patterns of syngeneic cytolytic T lymphocytes (CTL) directed against Gross MuLV-induced tumors were correlated with these quantitations of *H-2K^d* and *H-2D^d* antigens. Our results obtained by quantitative absorption of monospecific antisera indicated that the three BALB/c tumor cell lines expressed different amounts of *H-2K^d* and *H-2D^d* antigens, with *H-2D^d* antigen showing the greatest variability in expression because it ranged from barely detectable levels to one-eighth the amount of *H-2D^d* antigen expressed on normal BALB/c spleen cells. The *H-2* restriction patterns of Gross MuLV-specific CTL were directly affected by these quantitative modulations in the expression of *H-2K^d* and *H-2D^d* antigens, as revealed by three independent approaches: (a) inhibition of CTL activity by monospecific anti-*H-2* sera in the absence of complement; (b) competitive inhibition of CTL-mediated cytotoxicity by the addition of excess tumor cells into the reaction mixture; and (c) analysis of CTL specificities using cloned CTL populations. Our results thus indicate that *H-2* restriction of tumor-specific CTL activity can be directed at the target cell level by variations in the expression of *H-2* antigens.

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