VIRAL SPECIFICITY OF H-2-RESTRICTED T KILLER CELLS
DIRECTED AGAINST SYNGENEIC TUMORS INDUCED BY
GROSS, FRIEND, OR RAUSCHER LEUKEMIA VIRUS*

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Mouse cells infected by the Friend, Moloney, or Rauscher leukemia viruses (LV) express on their surfaces a cross-reactive antigen, FMR, which can be detected serologically (1). This antigen is absent from cells infected by Gross LV, a virus derived from leukemic AKR mice and showing genetic and structural relationships with Friend, Moloney, and Rauscher LV. Gross LV-infected cells express a Gross virus-associated cell-surface antigen (GCSA) which can be detected serologically and which does not cross-react with FMR antigen (2).

Studies of the cytolytic T lymphocyte (CTL) response in mice to syngeneic tumor cells induced by murine RNA LV have centered around LV of the FMR class. CTL sensitized against syngeneic tumor cells expressing the FMR antigen are capable of killing any syngeneic FMR-positive tumor cell; the same CTL, however, spare FMR-negative tumor cells induced by Gross LV, by chemical carcinogens, or by x rays (3–5). This pattern of specificity is parallel to that seen in serological studies, suggesting that the viral specificity of CTL involves the same antigen recognized by antibodies. However, the specificity of CTL generated in the mouse in response to syngeneic Gross LV-induced tumor cells has not been defined.

CTLs specific for FMR-positive tumor cells show a second specificity not detected in serological studies. This second specificity is directed against antigens encoded by the H-2K and/or H-2D genes of the major histocompatibility complex. Previous studies showed that FMR-specific CTL of the H-2b haplotype were effective only against tumor cells bearing the H-2D b antigen; CTL of the H-2d or H-2b haplotypes, however, appeared to be restricted to tumor cells bearing syngeneic H-2K antigens (6–8).

This communication concerns studies of the CTL response to syngeneic Gross LV-induced tumor cells. An earlier report by Meruelo et al. (9) indicated that a CTL response could be generated in mice against cells of a spontaneous lymphoma of the AKR strain, from which the Gross LV was originally derived. Our results indicate

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Abbreviations used in this paper: CTL, cytolytic T lymphocyte; FBS, fetal bovine serum; GCSA, Gross virus-associated cell-surface antigen; HSV, herpes simplex virus; LV, leukemia virus; MEM, Eagle's minimal essential medium; MLN, mesenteric lymph node; MLTC, mixed leukocyte-tumor cell cultures; PEC, peritoneal exudate cells; PLN, peripheral lymph node; TNP, 2,3,6-trinitrophenyl; VSV, vesicular stomatitis virus.
that a CTL response specific for syngeneic Gross LV-induced tumor cells could be elicited in mice of strains congenic to BALB/c but differing at H-2. The cytotoxic effector cells generated in vivo were diverse; however, effector-cell generation in mixed leukocyte-tumor cell cultures (MLTC) allowed us to selectively study the CTL response. These CTL were efficient in destroying syngeneic Gross LV-induced tumor cells, but not syngeneic FMR-positive tumor cells. Furthermore, their pattern of specificity for H-2K or H-2D antigens was somewhat different from that seen in parallel studies of FMR-specific CTL.

**Materials and Methods**

*Mice.* All mice were bred in our colonies at the Albert Einstein College of Medicine, Bronx, N.Y. The inbred strains used included C57BL/6 (B6, H-2b) and the series of H-2-congenic strains BALB/c (H-2d), BALB.B (H-2b), BALB.K (H-2k), BALB.G (H-2g), and BALB.5R (H-2r).

*Tumors.* Leukemia cells induced by Rauscher LV, Friend LV, Moloney LV, Gross LV, or chemical carcinogens were used. RBL-5 lymphoma cells (10), induced by Rauscher LV; MBL-2 lymphoma cells (10), induced by Moloney LV; EDG2 leukemia cells (2), induced by Gross LV; and EL4 leukemia cells (11), induced by benzpyrene, were derived in B6 mice (H-2b) and were maintained by serial passage in vivo. Friend LV-induced HFL/b cells (H-2b), derived from BALB.B mice (12), were maintained as stationary suspension cultures in Eagle's minimal essential medium (MEM) supplemented with 20% fetal bovine serum (FBS). Continuous cell cultures of tumors induced by Gross LV were derived in an analogous manner (12); newborn mice were injected i.p. with 0.1 ml of an acellular preparation of Gross LV derived from leukemic BALB/c mice. 2-3 mo later, the mice developed leukemia, and their enlarged spleens were minced into small fragments and injected i.v. into normal syngeneic adult mice. Single-cell suspensions were prepared from the resulting solid tumors and set in culture at various cell concentrations in MEM supplemented with 20% FBS. Continuous cell lines obtained in this manner from various strains of mice were: B.GV cells (H-2b, BALB.B), C.GV cells (H-2d, BALB/c), K.GV cells (H-2k, BALB.K), G.GV cells (H-2g, BALB.G), 5R.GV cells (H-2i5, BALB.5R), and B/CF1.GV cells (H-2b/a, [BALB.B × BALB/c]F1). Serological analysis (performed by Dr. E. Stockert of Sloan-Kettering Memorial Institute, New York) using B6 anti-K36 AKR leukemia serum showed that those lines tested (B.GV, C.GV, and K.GV) were GCSA-positive.

*Immunization Procedures.* Adult mice were given 10⁷ x-irradiated (5,000 rad) syngeneic tumor cells i.p. and allowed to rest for 20-40 d. These primed mice were (a) given a secondary s.c. or i.p. inoculum of 10⁶ untreated tumor cells, or (b) used as a source of leukocytes in MLTC.

*Leukocyte Suspensions.* Peritoneal exudate cells (PEC) were recovered by flushing the peritoneal cavity of each mouse twice with 5 ml MEM-5% FBS. Cell suspensions were prepared from the spleen, mesenteric lymph node (MLN), or the peripheral lymph nodes (PLN) by gentle disruption in a TenBroeck cell homogenizer (Fisher Scientific Co., Pittsburgh, Pa.). The leukocytes were suspended in 10 ml MEM-5% FBS, pelleted by centrifugation at 600 g for 5 min, and resuspended in 10 ml MEM-5% FBS. Debris was removed by sedimentation at unit gravity for 15 min. Each cell suspension was collected, pelleted at 600 g for 5 min, resuspended in MEM-5% FBS, and counted for viability.

*Lymphocyte Cultures.* LV-specific CTL were generated in MLTC from spleen cells of primed mice. Syngeneic MLTC were established in 20 ml RPMI-1640 medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 5% FBS and 5 × 10⁻⁵ M 2-mercaptoethanol, by mixing 2.5 × 10⁷ spleen cells with 1 × 10⁶ x-irradiated (5,000 rad) syngeneic tumor cells. The cells were harvested after 6 d in culture, washed, and counted for viability.

*⁵¹Chromium Release Cytotoxicity Assay.* Cell-mediated anti-tumor cytolytic activity was detected using a modification of the method of Brunner et al. (13). Varying numbers of viable lymphoid cells were mixed with 10⁴⁵¹Cr-labeled tumor cells in a final vol of 0.2 ml MEM-20% FBS. After 6 or 18 h incubation at 37°C, the supernates were harvested and measured for radioactivity. The percentage of specific ⁵¹Cr release was calculated for each lymphocyte to target cell ratio...
VIRAL AND H-2 SPECIFICITY OF CYTOLYTIC T LYMPHOCYTES

according to the following formula:

\[
\frac{ER - SR}{MR - SR} \times 100,
\]

where ER was the observed experimental \(^{51}\text{Cr}\) release; SR, the spontaneous release detected by incubating the target cells in culture medium alone; and MR, the maximum amount of radioactivity released from the target cells after incubation in 0.2 ml 1 N HCl for the duration of the assay. The spontaneous release (SR) values of the various tumor target cells used varied between 4 and 10% of the total incorporated label for 6-h incubation periods, and between 10 and 25% for 18-h incubation periods.

Cold Target Cell Inhibition Assay. CTL specificity for target antigens was analyzed by adding unlabeled competitor target cells to the \(^{51}\text{Cr}\) release cytotoxicity assay (4). Varying numbers of cold target cells were mixed with \(10^4\) \(^{51}\text{Cr}\)-labeled target cells in 0.1 ml culture medium; \(5 \times 10^5\) effector lymphocytes were then added in 0.1 ml to each well, and the plates were incubated for 6 h at 37°C. The percentage of inhibition of cytotoxicity was calculated according to the formula:

\[
\frac{\text{Cont.} - \text{Exp.}}{\text{Cont.}} \times 100,
\]

where Cont. was the specific cytotoxic activity detected in positive control wells containing effector lymphocytes and \(^{51}\text{Cr}\)-labeled target cells only; and Exp. was the experimental cytotoxicity values obtained from wells containing cold target cells, \(^{51}\text{Cr}\)-labeled target cells, and effector lymphocytes.

Macrophage Depletion with Carbonyl Iron. Macrophages and other phagocytic and/or adherent cells were removed from leukocyte suspensions by incubation of \(3 \times 10^7\) viable leukocytes with 40 mg carbonyl iron (Technicon Instruments Corp., Tarrytown, N. Y.) in 10 ml MEM-20% FBS at 37°C for 30 min with continuous shaking, followed by passage over a magnet (14).

Elimination of T Cells by AKR Anti-Thy 1.2 Serum. Aliquots of \(2 \times 10^7\) viable lymphoid cells were suspended in 1 ml MEM containing AKR anti-Thy-1.2 serum (10\(^{-1}\) final dilution), prepared by five successive immunizations of AKR mice with normal C3H thymus cells (15). After 30 min of incubation at 37°C, 2 ml MEM containing 10% rabbit serum (previously absorbed on agarose) was added to the cells as a source of complement, and the mixture was further incubated for 45 min at 37°C. After two washes in 10 ml MEM-5% FBS, the remaining cells were counted for viability and tested for cytotoxicity. Previous assays have indicated that this procedure results in the complete depletion of all T cells identifiable by immunofluorescent techniques (14).

Results

Generation In Vivo of Cytotoxic Cells in Response to Gross LV-induced Tumors. Tumors were induced by Gross LV in six strains of mice congenic to BALB/c at the H-2 region of chromosome 17 and expressing the homozygous haplotypes H-2\(^b\), H-2\(^d\), H-2\(^k\), H-2\(^e\), or H-2\(^p\), or the heterozygous haplotype H-2\(^b/d\). Each tumor was adapted to growth in vitro as a continuous line, and initial experiments showed that all cultures retained their tumorigenic potential in vivo when inoculated s.c. into syngeneic hosts (100% mortality at \(\leq 3\) wk after doses of \(10^5-10^6\) cells). However, at least 50% of the inoculated mice rejected their syngeneic tumor in each case when given \(10^4\) or fewer tumor cells. This indicated the existence of an immune response to these tumors, presumably triggered by a Gross LV-induced tumor antigen. In this context, serological typing indicated that the cells of each line tested were GCSA-positive.

A series of experiments was performed to examine the cell-mediated cytotoxic response to these Gross LV-induced tumors. Initial trials revealed the presence of cytolytic lymphoid cells in BALB.B mice after secondary immunization i.p., but not
FERNANDO PLATA AND FRANK LILLY

TABLE I

Time-Course of the Secondary Cytotoxic Response of BALB.B Mice to B.GV Tumor Cells

| Effector cells       | Percentage of specific ⁶⁷Cr release |
|----------------------|------------------------------------|
|                      | Day 1 | Day 3 | Day 5 | Day 8 |
|                      | 100:1 | 30:1  | 10:1  | 100:1 |
|                      | 10:1  | 30:1  | 10:1  |       |
|                      |       |       |       |       |
| MLN                  | 0     | 0     | 0     | 10    |
| PLN                  | 0     | 0     | 0     | 9     |
| Spleen               | 0     | 0     | 0     | 6     |
| Spleen + NMS + C*    | 0     | 5     | 6     | 4     |
| Spleen + anti-Thy 1.2 | 0     | 5     | 4     | 3     |
| PEC                  | 0     | 100   | 73    | 56    |
| PEC + NMS + C*       | 0     | 73    | 56    | 26    |
| PEC + anti-Thy 1.2 + C* | 0     | 73    | 56    | 26    |
| PEC + Fe⁺            | 0     | 73    | 56    | 26    |
|                      |       |       |       |       |

BALB.B mice were immunized with 10⁷ x-irradiated B.GV cells i.p. on day -27, and subsequently given a boost of 10⁶ untreated B.GV cells on day 0. Leukocytes were extracted from various lymphoid organs 1, 3, 5, and 8 d after the boost and assayed for lysis of ⁶⁷Cr-labeled B.GV cells in an 18-h cytotoxicity assay at three different lymphocyte to target-cell ratios.

* 2 × 10⁷ leukocytes were incubated with normal mouse serum (NMS) or AKR anti-Thy 1.2 serum and rabbit complement (C), washed twice, and assayed for cytolytic activity on B.GV cells.

† 3 × 10⁷ PEC were incubated with carbonyl iron (Fe) for 30 min at 37°C and then passed over a magnet to remove macrophages and other phagocytes.

s.c., with syngeneic B.GV cells. Table I shows that cytolytic cells capable of lysing B.GV cells in a long-term (18 h) ⁶⁷Cr-release assay could be recovered among MLN cells, spleen cells, and PEC. PLN cells isolated from the inguinal lymph nodes did not contain cytolytic cells. Table I also shows that PEC contained the highest number of cytolytic cells, and that the peak of activity occurred 3–5 d after the secondary boost. Other experiments indicated that cytotoxic activity dropped to negligible levels after day 10 in all tissues.

Attempts to identify these cytotoxic cells indicated that they were constituted in part by thymus-derived (i.e., T) cells, because incubation with anti-Thy-1.2 serum and rabbit complement induced a significant but incomplete abrogation of their lytic activity (Table I, day 5). Moreover, PEC cytotoxicity was also mediated by macrophages and/or other phagocytic cells, because cytolysis decreased after depletion of phagocytic and adherent cells with carbonyl iron (Table I, day 5).

Further experiments showed a general lack of specificity among cytolytic PEC generated in vivo. Table II shows that the lytic activity of these cells was specific neither for H-2 nor for viral antigens. Effector cells from BALB.B (H-2₅), BALB/c (H-2₆), and BALB.K (H-2₆) mice lysed tumor cells induced by both Gross LV and Friend LV, irrespective of their H-2 haplotype. These results indicated that Gross LV-induced tumor cells generated a cytotoxic response in vivo which was heterogeneous with respect to the nature of the killer cells and which lacked specificity.

Generation of Gross LV-specific CTL In Vitro. An experimental system was developed
TABLE II
Lack of Specificity of Cytotoxic PEC after Secondary In Vivo Immunization

| Target cells | Percentage of specific 51Cr release |
|--------------|-------------------------------------|
|              | BALB.B anti-B.GV | BALB/c anti-C.GV | BALB.K anti-K.GV |
|              | 100:1 30:1 10:1 | 100:1 30:1 10:1 | 100:1 30:1 10:1 |
|              | %                     | %                  | %                  |
| Gross LV-induced | | | |
| B.GV (H-2b) | 28 10 1 | 24 6 5 | 12 3 0 |
| C.GV (H-2d) | 22 4 0 | 50 19 2 | 6 3 3 |
| K.GV (H-2k) | 60 28 11 | 51 22 14 | 36 14 2 |
| Friend LV-induced | | | |
| HFL/b (H-2b) | 25 7 2 | 48 12 1 | 15 1 2 |
| HFL/d (H-2d) | 24 8 3 | 45 18 3 | 24 11 1 |
| HFL/k (H-2k) | 30 6 2 | 51 13 6 | 23 5 0 |

BALB.B, BALB/c, and BALB.K mice received 10⁷ x-irradiated B.GV, C.GV, or K.GV tumor cells, respectively, on day −36 and 10⁶ untreated tumor cells on day 0. On day 3 after the secondary boost, PEC from each group of mice were collected and assayed for cytotoxicity on a panel of 5Cr-labeled tumor cells in an 18-h cytotoxicity assay at various lymphocyte to target-cell ratios, as indicated.

which allowed the exclusive study of the CTL response elicited by immunization with Gross LV-induced tumor cells. This system involved the generation of CTL in syngeneic MLTC. Table III shows that cytotoxic cells, detectable in a 6-h 51Cr-release cytotoxicity assay, could be generated in MLTC by stimulating BALB.B spleen lymphocytes with B.GV cells. Spleens from either normal BALB.B mice (i.e., primary MLTC) or BALB.B mice primed with B.GV cells 30 d previously (i.e., secondary MLTC) could be used as sources of CTL precursor cells. The data in Table III show that secondary MLTC was 30 times more efficient than primary MLTC in generating CTL, because 3 × 10⁴ secondary MLTC cells yielded cytotoxicity comparable to 1 × 10⁶ primary MLTC cells (i.e., 11 and 18% specific 51Cr release, respectively).

Subsequent studies showed that cytolytic cells could also be generated in syngeneic secondary MLTC with spleen cells from BALB/c and BALB.K mice primed with syngeneic Gross LV-induced tumor cells (Table IV). Furthermore, each cytotoxic cell population was comprised essentially of T lymphocytes, because their effect could be eliminated by incubation with anti-Thy-1.2 serum in the presence but not the absence of complement (Table IV). Other experiments (data not shown) also indicated that macrophage depletion slightly increased, and did not inhibit, the cytotoxicity mediated by MLTC cells from BALB.B, BALB/c, or BALB.K mice. It thus became apparent that the cytolytic effector cells generated in our syngeneic secondary MLTC system and detected in 6-h cytotoxicity assays were of thymic origin.

Viral Specificity of CTL Generated in MLTC. A series of experiments was designed to study the capacity of CTL generated in MLTC to recognize diverse tumor antigens. CTL were generated in syngeneic secondary MLTC against H-2b tumor cells induced by Rauscher LV (i.e., B6 anti-RBL-5), Friend LV (i.e., BALB.B anti-HFL/b), and Gross LV (i.e., BALB.B anti-B.GV). Each CTL population was assayed on a panel of 5Cr-labeled H-2b tumor target cells which expressed FMR antigen (HFL/b, MBL-2, and RBL-5 cells) or GCSA (B.GV cells). Table V shows that B6 anti-RBL-5 and BALB.B anti-HFL/b CTL distinguished tumor cells expressing the FMR antigen...
Table III

Lysis of $^{51}$Cr-labeled B.GV Cells by BALB.B Lymphocytes Harvested from Primary and Secondary Syngeneic MLTC

| Source of killer cells | Percentage of specific $^{51}$Cr release |
|------------------------|----------------------------------------|
|                        | 100:1  | 30:1  | 10:1  | 3:1  | 1:1  |
| Primary MLTC           | 18     | 7     | 3     | 0    | 0    |
| Secondary MLTC         | 88     | 61    | 47    | 11   | 5    |

MLTC were established with $10^6$ x-irradiated B.GV cells and $25 \times 10^6$ responding lymphocytes from normal BALB.B mice (i.e., primary MLTC) or from BALB.B mice primed with $10^7$ x-irradiated B.GV cells 30 d earlier (i.e., secondary MLTC). After 6 d incubation, the lymphocytes recovered from MLTC were assayed for lytic activity against B.GV cells at various lymphocyte to target-cell ratios in a 6-h $^{51}$Cr-release assay.

from those expressing GCSA, because FMR-positive cells were lysed at least 10 times more efficiently than GCSA-positive cells. Conversely, BALB.B anti-B.GV CTL killed B.GV target cells at least 30 times more efficiently than any of the FMR-positive target cells. Consequently, CTL generated in syngeneic secondary MLTC were capable of selectively recognizing tumor target cells positive for FMR antigen or for GCSA. This conclusion was confirmed by other experiments, as shown in Fig. 1. It became apparent (Fig. 1 A) that BALB.B CTL generated against B.GV tumor cells in MLTC were capable of destroying EdG2 tumor cells ($H-2^b$, induced by Gross LV in B6 mice), as well as B.GV tumor cells, but not EL4 tumor cells ($H-2^k$, chemically induced in B6 mice). In addition, BALB.B CTL generated against HFL/b cells in MLTC (Fig. 1 B) killed FMR-positive HFL/b and RBL-5 target cells, but were incapable of destroying EdG2 cells, B.GV cells, or EL-4 cells. It should be stressed, however, that the observed viral specificities of CTL were not absolute, because low levels of cross-reactivity could sometimes be observed (Table V and Fig. 1).

Restriction by $H-2$ Antigens of Gross LV-specific CTL Activity. The $H-2$ transplantation antigens of the target cell had a restrictive effect on its recognition by Gross LV-specific CTL. The two parallel assays summarized in Fig. 2 concerned cold target cell competition of the lytic activity of Gross LV-specific CTL from BALB.B ($H-2^b$) and BALB/c ($H-2^a$) mice. A constant number of $10^4$ $^{51}$Cr-labeled B/CF1.GV tumor cells ($H-2^b/H-2^a$ heterozygous) were mixed with increasing numbers of unlabeled (i.e., cold) Gross LV-induced tumor cells of different $H-2$ haplotypes, and incubated with $5 \times 10^5$ MLTC cells. Lysis mediated by CTL from BALB.B mice ($H-2^b$) was efficiently inhibited by cold $H-2^{b/a}$ and $H-2^b$ Gross LV-induced tumor cells, and not by $H-2^d$ tumor cells (Fig. 2 A). On the other hand, CTL from BALB/c mice ($H-2^a$) were inhibited in their lytic activity by $H-2^{b/a}$ and $H-2^d$ Gross LV-induced tumor cells, but not by $H-2^b$ tumor cells (Fig. 2 B). When cold $H-2^b$ Gross LV-induced tumor cells were assayed with either set of CTL, a small degree of inhibition was observed with both $H-2^b$ and $H-2^d$ CTL at high doses of cold $H-2^k$ tumor cells. However, at least 10 times more cold $H-2^a$ tumor cells were required to reach the same degree of inhibition as observed with cold $H-2$-identical tumor cells. These data thus indicate that $H-2$ antigens played a role in the recognition of the target tumor antigens by Gross LV-specific CTL.

Attempts were made to extend these data by measuring the degree of CTL-mediated lysis of $^{51}$Cr-labeled tumor cells induced by Gross LV in congenic strains...
VIRAL AND H-2 SPECIFICITY OF CYTOLYTIC T LYMPHOCYTES

Table IV
Identification of Gross LV-specific Effector Cells From MLTC

| Effector cells | Target cells | No treatment | Anti-Thy 1.2 + MEM | Anti-Thy 1.2 + C |
|----------------|--------------|--------------|-------------------|-----------------|
|                |              | 30:1 | 10:1 | 3:1 | 1:1 | 30:1 | 10:1 | 3:1 | 1:1 | 30:1 | 10:1 | 3:1 | 1:1 |
| BALB.B anti-B.GV | B.GV         | 64   | 48   | 38 | 20 | 62 | 51 | 44 | 18 | 6   | 4   | 1   | 0   |
| BALB/c anti-C.GV | B/CF1.GV     | 15   | 5    | 3  | 1  | 21 | 9  | 4  | 2  | 4   | 1   | 0   | 0   |
|                 | G.GV         | 30   | 9    | 2  | 4  | 42 | 12 | 4  | 2  | 0   | 0   | 0   | 0   |
| BALB.K anti-K.GV | K.GV         | 21   | 6    | 2  | 1  | 20 | 10 | 6  | 2  | 7   | 6   | 4   | 0   |

Gross LV-specific effector cells were generated in syngeneic secondary MLTC, harvested on day 6 after culture initiation, and counted. Aliquots of 2 × 10⁶ viable lymphocytes were incubated with AKR anti-Thy-1.2 serum in the absence or presence of rabbit complement, washed twice, recounted, and assayed for cytotoxicity at various lymphocyte to target-cell ratios in a 6-h ⁵¹Cr-release assay.

 bearing H-2-recombinant haplotypes. Table VI summarizes the results obtained with tumor cells of the H-2a haplotype (H-2KdD b, G.GV cells) and H-2b haplotype (H-2KbD b, 5.R.GV cells). BALB.B anti-B.GV CTL (H-2KbD b) were efficient killers of all Gross LV-induced target cells expressing either H-2K b or H-2D b antigens, or both. There was no absolute requirement for the expression of either H-2K b or H-2D b antigens, because the single expression of H-2K b or H-2D b antigen was sufficient for lysis. In agreement with results shown in Fig. 2, Gross LV-induced C.GV tumor cells from BALB/c mice (H-2KbD a) were not killed by BALB.B CTL; K.GV tumor cells from BALB.K mice (H-2KbD b) were lysed only to a minimal extent (30 times less efficiently than B.GV cells from BALB.B mice).

Analogous assays with Gross LV-specific CTL from BALB/c mice (H-2KbD b) revealed a different pattern of restriction by H-2: as shown in Table VI, H-2K d antigens seemed to be necessary and sufficient for recognition of Gross LV-induced tumor antigens. The presence of H-2D b antigens (in the absence of H-2K b) was not sufficient, because 5.R.GV tumor cells (H-2K bD b) were poorly lysed. BALB/c CTL were incapable of killing B.GV tumor cells (H-2K bD b), and killed K.GV tumor cells (H-2K bD b) to a minimal extent. Furthermore, although BALB/c anti-C.GV CTL efficiently lysed B/CF1.GV hybrid tumor cells (H-2K d), they were incapable of lysing C.GV cells from BALB/c mice. The reason for this inability to lyse C.GV cells is not understood, because C.GV cells induced the generation of H-2d-restricted CTL in MLTC, inhibited the lysis of B/CF1.GV tumor target cells by BALB/c anti-C.GV CTL (Fig. 2 B), and were susceptible to lysis by BALB.B (H-2b) anti-H-2 d CTL (not shown). This peculiar resistance to lysis manifested by C.GV cells is not a general characteristic of Gross LV-induced tumor cells in BALB/c mice: we have recently established two new BALB/c-GV lines which proved to be susceptible to Gross LV-specific CTL from BALB/c mice (data not shown), when assayed shortly after establishment as continuous cell lines.

It was difficult to generate Gross LV-specific CTL in MLTC from BALB.K (H-2KbD b) immune spleen cells. As shown in Table IV and VI, the cytotoxic activity was low, with values rarely surpassing 25% specific ⁵¹Cr release at 100:1 lymphocyte to target cell ratios. These low levels of cytolytic activity, however, were mediated by T
cells (Table IV) and were restricted to BALB.K Gross LV-induced tumor cells (Table VI). This finding was in accordance with the observation that $H-2^b$ mice are highly susceptible to leukemia induction by Gross LV, as compared with $H-2^b$ and $H-2^d$ mice (16).

**Discussion**

These data indicate the existence of a heterogeneous and dynamic cell-mediated immune response to Gross LV-induced tumors. Earlier studies (16) had revealed an $H-2$-linked immune resistance to leukemia induction in neonates by Gross LV, such that $H-2^b$ mice proved to be resistant to Gross LV, $H-2^b$ mice were highly susceptible, and $H-2^d$ mice were intermediate. Further analyses showed that susceptibility or resistance to Gross LV was governed in part by a single immune response gene, $R_{gv-1}$, mapped to the $K$ or $I$ region of $H-2$ (17).

The immune resistance to Gross LV-induced leukemia is probably mediated by a variety of active cells, including T and non-T cells. When we studied the secondary response in vivo of BALB.B mice to the syngeneic Gross LV-induced tumor B.GV, we found evidence for the existence of cytotoxic T cells and phagocytic non-T cells (probably macrophages) among PEC. These cells were particularly active between days 3 and 5 after a secondary immunization with B.GV cells; however, they did not show obvious $H-2$ or tumor specificities in an in vitro cytotoxicity assay, probably a result of the presence of nonspecific cytotoxic macrophages. Gomard and colleagues (18) previously reported the existence of similar cytotoxic macrophages in preleukemic AKR mice, indicating that Gross LV (endogenous in AKR mice) might be particularly effective in activating macrophages.

Our preliminary aim, however, was to study the specificity of the CTL subpopulation present among the cytotoxic cells elicited in response to Gross LV-induced tumors. Previous reports had indicated that Gross LV-specific CTL could be generated in rats (19, 20). Consequently, it was particularly interesting to consider murine Gross LV-immune CTL with respect to their tumor specificity and to their specificity for particular $H-2$ antigens, and to compare them with the specificities of CTL immune to FMR-positive tumor cells. For this purpose we resorted to the generation of tumor-specific CTL in vitro. CTL generated in syngeneic MLTC could readily be detected in a $^{51}$Cr-release cytotoxicity assay and possessed a high degree of specificity for the immunizing LV-induced tumor antigens.

**Table V**

**Viral Specificity of $H-2^b$ CTL Generated in Syngeneic Secondary MLTC**

| Target cells | Percentage of specific $^{51}$Cr release (in vitro) |
|--------------|---------------------------------------------------|
|              | BALB.B anti-HFL/b | BALB.B anti-B.GV |
| B6 anti-RBL-5 | B6 anti-RBL-5 | B6 anti-RBL-5 |
| 100:1 | 30:1 | 10:1 | 3:1 | 1:1 | 100:1 | 30:1 | 10:1 | 3:1 | 1:1 |
| HFL/b (Friend LV) | 32 | 20 | 8 | 4 | 0 | 23 | 18 | 7 | 2 | 0 | 5 | 1 | 0 | 0 | 0
| MBL-2 (Moloney LV) | 41 | 26 | 12 | 6 | 0 | 51 | 27 | 20 | 8 | 5 | 12 | 2 | 0 | 0 | 0
| RBL-5 (Rauscher LV) | 56 | 35 | 10 | 6 | 0 | 58 | 37 | 30 | 14 | 5 | 9 | 6 | 2 | 0 | 0
| B.GV (Gross LV) | 11 | 9 | 5 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 63 | 47 | 19 | 12 | 4

Lymphocytes were harvested after 6 d of incubation in MLTC, counted, and tested for cytotoxicity against a panel of $H-2^b$ $^{51}$Cr-labeled target cells at the indicated lymphocyte to target-cell ratios in a 6-h assay.
Specificity studies showed that Gross LV-immune CTL, although recognizing and destroying syngeneic Gross LV-induced tumor cells efficiently, were incapable of killing syngeneic FMR-positive tumor cells. Syngeneic leukemia cells induced by chemical carcinogens were also spared. Conversely, CTL generated in response to syngeneic FMR-positive tumor cells were very poor effectors against syngeneic Gross LV-induced tumor cells. These patterns of CTL tumor specificity were found to be true for mice of the \(H-2^b\) haplotype (i.e., BALB.B, Table V) and of the \(H-2^d\) haplotype (i.e., BALB/c, data not shown). Mice of the \(H-2^b\) haplotype (i.e., BALB.K) consistently gave weak but specific CTL responses to syngeneic Gross LV-induced tumor cells. The magnitude of these CTL responses paralleled the relative resistance to leukemia induction by Gross LV in \(H-2^b\) and \(H-2^d\) mice, as opposed to the high degree of susceptibility in \(H-2^k\) mice, attributed to effects of the \(Rga-1\) gene.

The tumor specificity evident among Gross LV-specific CTL was accompanied by specificity for \(H-2\) antigens; i.e., \(H-2\) restriction. Attempts were made to map this restriction to the \(H-2K\) or \(H-2D\) locus of \(H-2^b\) and \(H-2^d\) mice by using tumor target cells induced by Gross LV in BALB.G (\(H-2K^dD^b\)) and BALB.5R (\(H-2K^bD^d\)) \(H-2\)-recombinant mice. Our results indicated that \(H-2^b\) CTL from BALB.B mice recognized Gross LV-induced tumor antigens in association with \(H-2^d\) determinants coded by either \(H-2K^b\) or \(H-2D^b\) genes. However, \(H-2^d\) CTL from BALB/c mice were restricted to recognition of Gross LV-induced tumor antigens in association with \(H-2K^a\); association with \(H-2D^a\) did not lead to significant killing by CTL. We were unable to map the \(H-2\) restriction of \(H-2^b\) Gross LV-specific CTL as a result of their low and inconsistent cytolytic activities.

Our observations on the \(H-2\) restriction of Gross LV-specific CTL can be compared with those made in other systems of CTL immunity concerning tumors induced by Friend and Moloney LV; whereas \(H-2^b\) Gross LV-specific CTL were effective in the presence of either \(H-2K^b\) or \(H-2D^b\), \(H-2^b\) Friend LV-specific (7) and Moloney LV-specific (8) CTL showed a clear preference for tumor antigens associated with \(H-2D^b\), and not \(H-2K^b\). \(H-2^d\) mice were very poor responders to Friend LV-induced tumor cells (6–8). However, the same mice responded well to Moloney LV-induced antigens.
H-2 specificity of Gross LV-immune CTL revealed by competitive inhibition of cytotoxicity using cold target cells. BALB.B anti-B.GV CTL (H-2b, panel A) and BALB/c anti-C.GV CTL (H-2d, panel B) were generated in MLTC and tested for cytotoxicity in a 6-h assay on 10^4 ^51Cr-labeled B/CFt.GV target cells (H-2b/d) at a ratio of 50 lymphocytes to 1 target cell. The average ^51Cr release values were 31% for BALB.B anti-B.GV CTL and 33% for BALB/c anti-C.GV CTL. The following unlabeled Gross LV-induced tumor cells were added at various doses to inhibit cytotoxicity in a selective manner: B/CFt.GV cells (Δ, H-2b/d), K.GV cells (▲, H-2k), C.GV cells (○, H-2a), and B.GV cells (●, H-2b). The percentage of specific inhibition of cytotoxicity was calculated for each dose of cold tumor cells added, as described in Material and Methods.

An intriguing paradox raised during the course of our studies was the resistance of C.GV tumor cells to lysis by Gross LV-immune CTL. C.GV cells, induced by Gross LV in BALB/c mice, were susceptible to lysis by BALB.B (H-2b) anti-BALB/c (anti-H-2^d) CTL. Moreover, C.GV cells could induce the generation of Gross LV-immune CTL in MLTC, and could effectively block the lysis of H-2^b/d Gross LV-induced tumor cells mediated by Gross LV-immune CTL from BALB/c mice. Furthermore, two new Gross LV-induced tumor cell lines from BALB/c mice, tested in early passages in culture, were susceptible to attack by BALB/c anti-C.GV CTL, and could also induce CTL generation in syngeneic MLTC (data not shown). During the course of other studies, we observed similar patterns among cells derived from a Friend LV-induced tumor, in the sense that certain clones of the HFL/b tumor cell line were more susceptible than others to attack by Friend LV-specific CTL; however, those clones which proved to be poor targets to CTL attack were excellent stimulators in MLTC. Experiments are currently in progress in our laboratory to determine whether these patterns also apply to Gross LV-induced cell lines and to other LV-induced tumors.

The data presented in this report thus provide further evidence for the fine specificity of recognition and activity of cytolytic T lymphocytes. CTL have been shown to recognize minor antigenic changes induced by point mutations in H-2 gene structure in various models of immunity (21-23).
Table VI

| Target cells                | BALB.B anti-B.GV CTL (H-2^d) | BALB/c anti-C.GV CTL (H-2^d) | BALB.K anti-K.GV CTL (H-2^d) |
|-----------------------------|-------------------------------|-------------------------------|-------------------------------|
|                            | 30:1 10:1 3:1 1:1             | 30:1 10:1 3:1 1:1             | 100:1 30:1 10:1 3:1           |
| B.GV (H-2K^dD^d)           | 54 44 26 15                  | 5 1 0 0                      | 3 0 0 0                       |
| C.GV (H-2K^dD^d)           | 0 0 0 0                      | 2 0 0 1                      | 4 1 0 0                       |
| B/CF1.GV (H-2K^dD^dD^K^K)   | 33 20 10 3                   | 48 37 25 7                   | 5 2 2 1                       |
| K.GV (H-2K^dD^d)           | 9 2 0 0                      | 11 9 3 3                     | 26 7 3 1                      |
| G.GV (H-2K^dD^d)           | 25 16 11 6                   | 53 34 25 21                  | 5 0 0 0                       |
| 5R.GV (H-2K^dD^d)          | 48 36 24 15                  | 17 1 0 0                     | 0 0 0 0                       |

Specific cytotoxicity was determined in a 6-h \(^{51}Cr\) release assay at the indicated lymphocyte to target-cell ratios.

Antigenic differences. CTL sensitized to syngeneic cells infected with vesicular stomatitis virus (VSV), for example, not only could distinguish among target cells infected with different strains of VSV, but also could distinguish among cells infected by different temperature sensitive mutants of VSV (24). Similarly, CTL sensitized to syngeneic cells infected with herpes simplex virus (HSV) could distinguish among targets infected with HSV type 1 and HSV type 2 (25), and CTL generated against influenza virus were specific for distinct categories of influenza virus hemagglutinin (26). Finally, CTL generated in response to syngeneic lymphocytes coupled to the hapten 2,3,6-trinitrophenyl (TNP) destroyed syngeneic target cells coupled to TNP, but not those cells which were coupled to dinitrophenyl (27), to other nitrophenyl compounds (28), or to p-azophenylarsonate (29), and vice versa. Our results are in accord with the high degree of virus and H-2 specificity seen in these experiments. The serologically established distinction between the FMR and the GCSA categories of murine LV are clearly maintained in our studies of the CTL response to LV-induced tumors.

Summary

Cytolytic T lymphocytes (CTL) were generated against murine tumors induced by Gross, Friend, or Rauscher leukemia virus (LV) in syngeneic mixed leukocyte-tumor cell cultures. Analogous to the patterns of specificity observed with antibodies to LV-induced cell surface antigens, CTL could be classified into two major groups of specificity. Tumor cells induced by Friend, Moloney, or Rauscher virus and positive for the FMR antigen were killed by syngeneic CTL immune to any one of these three LV; the same CTL, however, were incapable of killing syngeneic tumor cells induced by Gross LV. The converse was true for Gross LV-specific CTL: these CTL were specific for syngeneic tumor cells expressing the Gross virus-associated cell-surface antigen (GCSA), and not the FMR antigen. The H-2 specificities of the two groups of LV-immune CTL were also compared, because in both cases, CTL were restricted in their killing activity to H-2-identical tumor target cells. When CTL from single strains of mice were generated against syngeneic FMR- or GCSA-positive tumor cells, differences were observed with respect both to the requirement for the expression of
compatible H-2K or H-2D specificities, and to the intensity of the CTL response in congenic mice of the H-2^b, H-2^d, and H-2^k haplotypes.

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1186 VIRAL AND H-2 SPECIFICITY OF CYTOLYTIC T LYMPHOCYTES

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