STUDIES ON THE ROLE OF THE HOST IMMUNE RESPONSE IN RECOVERY FROM FRIEND VIRUS LEUKEMIA

II. Cell-Mediated Immunity

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Cell-mediated immune phenomena appear to play a major role in host defense against antigenically foreign cells such as allografts and tumors. Passive transfer of immune lymphoid cells can often accelerate allograft rejection (1, 2) or protect specifically against challenge with neoplastic cells (3, 4). In the case of mouse leukemia and sarcoma virus-induced tumors several different cell-mediated immune mechanisms have been demonstrated in vitro (see references 5–7 for review). However, the in vivo roles and relative importance of these mechanisms remain unclear at this time.

Friend virus (FV)[1] mouse leukemia presents an excellent model for investigation of host defenses involved in recovery from primary virus-induced neoplasia. The onset of disease is very rapid and immunologically mature adult mice of many strains are susceptible to induction of the leukemia. Furthermore, several genes have been identified that influence FV leukemia including one (*Rfv-1*) which appears to affect recovery from established leukemia (8–11). The possible role of the host immune response in the mechanism of action of this and other *H-2*-associated genes influencing mouse leukemia has been postulated (9). The previous paper in this series presented data on the humoral immune response to FV and FV-induced leukemia cells (12). This paper will examine some aspects of the specific cell-mediated immune response in this disease. The results indicate that θ-positive cells, which are specifically cytotoxic for FV leukemia cells in vitro, appear in the spleens of mice during or after spontaneous recovery from FV-induced splenomegaly. Mice of both high and low recovery (*Rfv-1*) genotypes can produce these effector cells, and thus the *Rfv-1* gene does not seem to function by means of an absolute control over production of these specific immune cells. The virus-induced antigens detected by the cytotoxic cells in this system appear to be different than those detected with serological techniques. Furthermore, the *H-2* type of the leukemia cells seems to play a role in expression of these antigens.

[1] *Abbreviations used in this paper:* ADNCC, antibody-dependent normal lymphoid cell-mediated cytotoxity; B/T-L, BALB Tennant leukemia virus; CMC, cell-mediated cytotoxicity; FFU, focus-forming units; FV, Friend leukemia virus; LCM, lymphocytic choriomeningitis; MolLV, Moloney leukemia virus; MSV, Moloney sarcoma virus; RLV, Rauscher leukemia virus.

Materials and Methods

Mouse strains, buffers, virus preparations, virus assay, and spleen palpation techniques were described previously (11, 12). In addition, B10.A(18R) mice were provided by Dr. J. Stimpfling, Great Falls, Montana. These mice have the *b* allele of the *H-2K* region and the *d* allele of the *H-2D* region.[2] The F_1 produced by mating these mice with A.BY mice has the *Rfv-1*$^{d/b}$ genotype with a pattern of recovery from FV-induced splenomegaly identical to (B10.A × A.BY)F_1 mice.

Lymphoma Cell Lines. A13, BB4, BC1, BC2, and BC-3A tumor lines were induced by i.p. inoculation of 1–6-day-old mice with BALB Tennant leukemia (B/T-L) virus. B/T-L virus was selected because it is the helper MuLV for the B-tropic FV stock used (13), and thus these viruses have common envelope antigens. A13 and BB4 were from BALB.B (*H-2*$^{b/b}$)mice, and BC1, BC2, and BC-3A were from BALB/c (*H-2*$^{d/d}$) mice. Mice developed leukemia 2–5 mo after virus inoculation, and lymph node cell suspensions were passed i.p. to syngeneic mice. Usually the cells grew in the spleen and lymph nodes; however, after repeated i.p. passages, cells grew freely throughout the peritoneal cavity and were maintained as ascites lines. T2, T4, T5, and HFL/d-sc tumor lines were derived from BALB/c mouse spleens 50-70 days after inoculation with NB-tropic FV. Spleen fragments (1–3 mm) were injected subcutaneously in BALB/c recipients. Subcutaneous tumors grew in 10–20 days, and cells were passed i.p. as ascites cells thereafter. HFL/d-sc was produced in the laboratory of Dr. F. Lilly, Albert Einstein School of Medicine, Bronx, New York and was provided to us in the 49th subcutaneous passage (14, 15). FBL-3 is a C57BL/6 lymphoma induced by the lymphatic leukemia helper virus of the FV complex. It was provided by Dr. A. Fefer of the University of Washington School of Medicine, Seattle, Wash. LSTRA is a BALB/c lymphoma induced by Moloney leukemia virus and was provided by Dr. J. Wunderlich, National Cancer Institute, Bethesda, Md. RBL-5 and EL4(G-) are both C57BL/6 lymphomas and were provided by Dr. R. Herberman, National Cancer Institute, Bethesda, Md.

Ascites cells were also grown in vitro as stationary suspension cultures in RPMI 1640 with 10% fetal calf serum, 200 U/ml penicillin, and 10 μM 2-mercaptoethanol. Cells were inoculated at an initial density of 5–10 × 10^4 cells/ml and usually grew to a maximum concentrations of 2–4 × 10^6/ml within 3–5 days, at which time cells were passed by direct transfer of the appropriate number of cells into a new flask with fresh medium.

58 is an FV-induced cell line from the leukemic spleen of a (B10.A(4R) × A.BY)F_1 mouse. Spleen cells were grown directly in vitro in stationary suspension cultures and passed thereafter as described above. These cells have only been propagated in vitro, and numerous attempts to grow them in vivo have been unsuccessful.

Cell-Mediated Cytotoxicity (*CMC*). A microadaptation of the assay of Brunner et al. was employed (16). Immune and normal "attacker" spleen cells were dissociated in 12 ml phosphate-buffered balanced salt solution (PBBS), and large clumps were removed by decanting. Suspensions were centrifuged 1,500 rpm for 5 min, cell pellets were resuspended in RPMI 1640 medium, as described above, and the number of viable nucleated cells was determined. The concentration was adjusted to 13.3 × 10^6/ml and 0.15 ml (2 × 10^6 cells) was pipetted into quadruplicate wells of Linbro sterile flat bottomed microtiter trays (Linbro Chemical Co., New Haven, Conn.). Target lymphoma cells were labeled with ^{51}Cr as described (12), washed three times, and resuspended in medium at a concentration of 4 × 10^5 cells/ml. 0.025 ml (1 × 10^4 cells) was delivered into each well with a dropper, and trays were then incubated for 19–20 h in 5% CO_2 in air at 34°C.[3] After incubation the trays were centrifuged 1,000 rpm for 5 min, and 0.100 ml of supernate was removed from each well and counted in an automatic gamma counter. Control wells contained normal spleen cells, and percent cytotoxicity was calculated as:

$$\frac{\text{cpm immune spleen} - \text{cpm normal spleen}}{\text{total cpm targets} - \text{cpm normal spleen}} \times 100$$

When inhibition assays (17) were performed, attacker spleen cells were used at a concentration of 20 × 10^6/ml, and 0.100 ml (2 × 10^6 cells) was added to each well. Inhibitor cells or sera were used

[2] Stimpfling, J. 1975. Personal communication.

[3] Although the CMC also worked at 37°C, 34°C was found empirically to give optimal specific cytotoxicity due to a reduction in nonspecific ^{51}Cr release and no significant decrease in immune cell-mediated ^{51}Cr release.

at various concentrations and were added to wells in a volume of 0.050 ml. Targets were then added by dropper as before. Assay of antibody-dependent normal lymphoid cell-mediated cytotoxicity (ADNCC) (18) was done in a similar fashion to the inhibition CMC assay. In some experiments 15,000 focus-forming units (FFU) of B-tropic FV (C57BL/10 × A.BY)F_1 stock) was added to the wells to check for possible inhibition of CMC.

Irradiation. Attacker cells were given 2,000 R at 31 rads/min using a GE Maximar X-ray unit at 250 kV, 15 mA, with 1-mm Cu and 1-mm Al filters.

Antisera. Anti-θ serum was prepared as described by Reif and Allen (19). Serum was absorbed with θ-negative BALB/c lymphoma cells before use. When tested for cytotoxicity using absorbed rabbit serum as complement (C′), absorbed anti-θ serum killed θ-positive mouse lymphoma cells (EL4, A13, BB4), and did not kill known θ-negative mouse lymphoma cells (FBL-3, LSTRA, BC1). When tested against normal (C57BL/10 × A.BY)F_1 spleen cells 40–50% cytotoxicity was observed.

Anti-Ia8 serum was prepared in (B10.A × A/WySn)F_1 mice by immunizing with B10.D2 tail skin grafts followed by booster injections of spleen cells (20–40 × 10^6 cells i.p.) at 14-day intervals. Mice were bled after 3–6 injections. Similar to results reported with B10.A anti-B10.D2 serum (20), our anti-Ia8 serum in the presence of rabbit C′ killed 40–50% of normal C57BL/10 or (C57BL/10 × A.BY)F_1 spleen cells, >95% of several Ia8-positive lymphomas and 0% of an Ia8-negative lymphoma (EL4). (Lymphomas were typed for presence of Ia8 using direct cytotoxicity with anti-Ia8 sera kindly provided by Dr. D. Sachs, National Cancer Institute, Bethesda, Md.)

(C57BL/10 × A.BY)F_1 and (B10.A × A.BY)F_1 anti-FV serums were from mice inoculated i.v. with 15,000 FFU B-tropic FV stock from syngeneic mice. Mice that recovered from splenomegaly were bled 30–50 days later.

H-2^d anti-*H-2^b* serum was obtained by immunizing (B10.D2 × BALB/c)F_1 mice with C57BL/10 tail skin grafts followed by i.p. booster inoculation of spleen cells at 14-day intervals. Mice were bled 10 days after the fourth inoculation.

Anti-θ and Anti-Ia8 Treatment of Spleen Cells. Diluent was PBBS with 5% fetal calf serum. 40 × 10^6 attacker spleen cells were incubated with 0.125 ml anti-θ serum or 0.2 ml anti-Ia8 serum or similar amounts of normal mouse serum in a total volume of 2 ml for 30 min at 37°C. Cells were then washed twice, resuspended in a 1.0-ml volume, and 1 ml of a one:eight dilution of absorbed rabbit serum (12) was added as C′ source. Cells were incubated 45 min at 37°C, washed twice, and viable nucleated cells counted. Cells were then tested as usual in the CMC assay.

Removal of Adherent Cells from Spleen Populations. Glass-adherent cells were removed from attacker spleen suspensions using columns of glass beads (Superbrite, type 100-5005, Minnesota Mining and Manufacturing Co., St. Paul, Minn.). 200 × 10^6 cells were absorbed for 20 min at 37°C on a 15-ml column of beads in PBBS with 5% fetal calf serum, and unattached cells were then eluted, washed, and used in the CMC assay. Recovery was usually 10–25% of the original cell population. In two experiments (no. 1 and 2, Table V) glass-adherent cells were removed by incubation of 40 × 10^6 attacker spleen cells in 20-ml medium at 37°C in 1 liter prescription bottles (110 cm^2 absorption surface). Unattached cells (30% of original cell population) were decanted after 90 min and used in the CMC assay. No phagocytosis of latex particles was seen in the nonadherent cell populations obtained by either of these methods.

Results

Correlation of CMC with Regression of FV-Induced Splenomegaly. Spleen cells from mice of various strains previously inoculated with FV were tested as attackers in the CMC assay using A13 target cells. Spleen cells from both high recovery, *Rfv-1$^{b/b}$*, mice [(C57BL/10 × A.BY)F_1, (B10.A(1R) × A.BY)F_1, (B10.A(2R) × A.BY)F_1, (B10.A(4R) × A.BY)F_1 and (C57BL/10 × BALB.B)F_1] and low recovery, *Rfv-1$^{d/b}$*, mice [(B10.A × A.BY)F_1, (B10.A(5R) × A.BY)F_1 and (B10.A(18R) × A.BY)F_1] were observed to produce significant cytotoxicity (Table I). With all genotypes, there was a very strong correlation between recovery from splenomegaly (regressor mice) and significant CMC. Of mice with splenomegaly (progressor mice), only 2 of 26 were positive in the CMC assay. Both of these individuals had borderline increases in spleen size and could possibly have

TABLE I
Correlation of CMC with Regression of FV-Induced Splenomegaly

CMC‖	*Rfv-1* genotype					
	*b/b**		*d/b*‡		*b/b*§	
	>5%	≤5%	>5%	≤5%	>5%	≤5%
Regressor mice¶	61	10	19	3	7	1
Progressor mice	0	8	2	9	0	7

* Includes $Rfv\text{-}1^{b/b}$ mouse strains (C57BL/10 × A.BY)F_1, (B10.A(1R) × A.BY)F_1, (B10.A(2R) × A.BY)F_1, and (B10.A(4R) × A.BY)F_1 given 15,000 FFU FV i.v. Spleens were assayed 4–34 days after virus inoculation. P value <0.001.

‡ Includes $Rfv\text{-}1^{d/b}$ mouse strains (B10.A × A.BY)F_1, (B10.A(5R) × A.BY)F_1, and (B10.A(18R) × A.BY)F_1 given 15,000 or 1,500 FFU FV i.v. Spleens were assayed 10–41 days after virus inoculation. P value <0.001.

§ (C57BL/10 × BALB.B)F_1 mice ($Rfv\text{-}1^{b/b}$) given 300 FFU FV i.v. Spleens were assayed 14–46 days after virus inoculation. P value <0.001.

‖ Cell-mediated cytotoxicity against ^{51}Cr-A13 target cells. Values of greater than 5% specific cytotoxicity were considered positive.

¶ Criteria for regression of splenomegaly were described in reference 11.

already initiated recovery mechanisms. This discrepancy probably represents the inaccuracy inherent in spleen palpation as a measure of disease state. The incidence of regression seen in Table I for low recovery genotype mice ($Rfv\text{-}1^{d/b}$) does not represent a typical population incidence, since mice without splenomegaly were preferentially selected to demonstrate their ability to produce CMC. In addition, a lower virus dose (1,500 FFU) was given to many of these mice to increase the incidence of recovery (11) and provide regressor spleens for the CMC assay.

There was a wide range of percent cytotoxicity observed with all mouse strains studied. The data for individual mice of high and low recovery *Rfv-1* genotypes suggest that both genotypes can produce CMC of equal magnitude when tested at the optimal time during or after recovery from splenomegaly (Fig. 1 and 2). Peak CMC tended to occur between 10 and 35 days after virus inoculation, and this coincided with the usual time of regression of splenomegaly in these strains at the virus doses studied. Later than 35–40 days postinoculation most CMC values were near or below 5%, which was generally the area of borderline statistical significance. There was a small number of mice (10 of 71 in Fig. 1 and 3 of 22 in Fig. 2) that gave 5% or lower CMC even though they were examined at the apparent optimum time after recovery from splenomegaly. The reason for this finding is unclear. These mice could have been on the verge of a relapse of splenomegaly, as sometimes occurs, or possibly their spleens still contained enough neoplastic cells to compete with the ^{51}Cr-labeled targets in the in vitro assay.

Specificity of the CMC. As shown above, attacker spleen cells from (C57BL/10 × BALB.B)F_1 mice could lyse A13 (BALB.B) target cells effectively. Since BALB.B is one of the parents of these hybrid mice, there should be no reaction of these attackers directed to normal nonviral-induced antigens of these

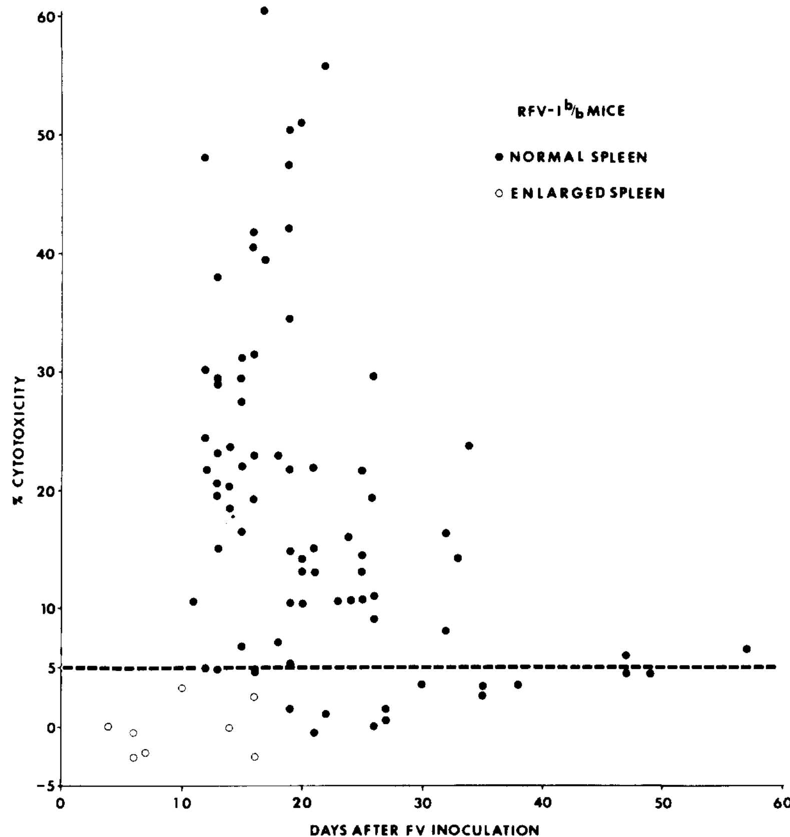

FIG. 1. CMC values obtained using attacker spleen cells from individual *Rfv-1*$^{b/b}$ (high recovery incidence) mice [(C57BL/10 × A.BY)F_1, (B10.A(1R) × A.BY)F_1, (B10.A(2R) × A.BY)F_1, and (B10.A(4R) × A.BY)F_1] tested against ^{51}Cr-labeled A13 target cells at various times after i.v. inoculation of FV (15,000 FFU (C57BL/10 × A.BY)F_1 stock). Greater than 5% cytotoxicity was considered significant.

target cells. However, with the A.BY hybrid congenic series of attackers, targets and attackers in the CMC assay were not always identical for cell membrane transplantation antigens, and it was important to investigate the specificity of the CMC that was observed. As noted in the methods, virus stocks used for inoculation were from spleens of syngeneic mouse strains and therefore should contain no immunizing nonviral antigens. To further examine the specificity of the CMC observed, inhibition experiments were performed using unlabeled spleen cells from normal mice and mice with FV-induced splenomegaly as competitors against the ^{51}Cr-labeled A13 target cells. Spleen cells from mouse strains (C57BL/10 × BALB.B)F_1, (C57BL/10 × A.BY)F_1, (B10.A × A.BY)F_1, and (B10.A × A/WySn)F_1 were tested for inhibition of CMC using (C57BL/10 × A.BY)F_1 attackers. The results are shown in Fig. 3. For each inhibitor strain tested the normal spleen cells gave no inhibition of CMC, whereas the FV leukemic spleen cells gave a significant concentration-dependent depression of CMC. Identical results were obtained using (B10.A × A.BY)F_1 attacker cells. These observations suggest that in this system the lysis obtained is directed

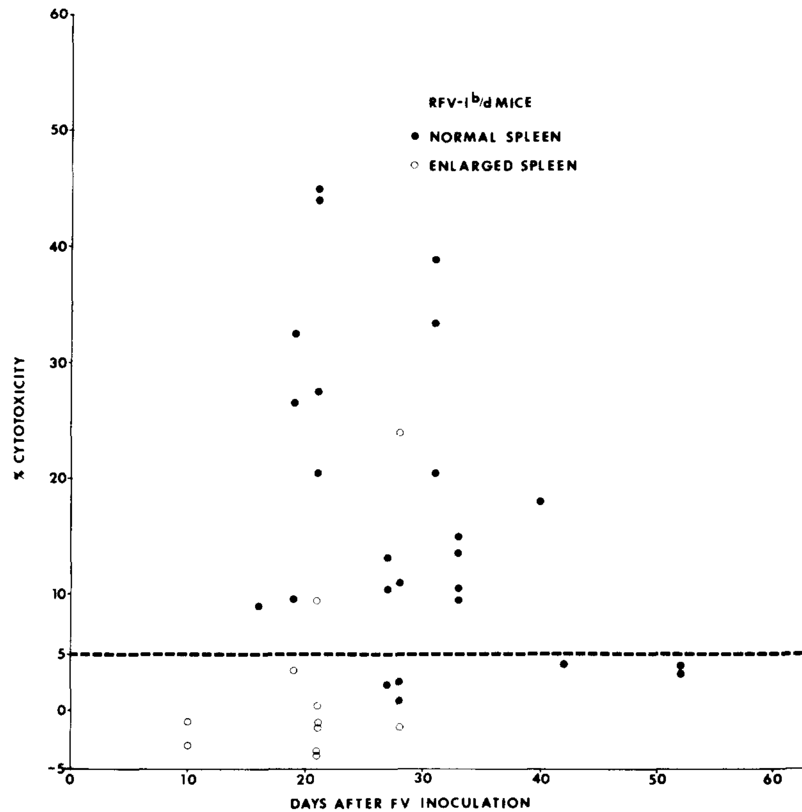

FIG. 2. CMC values obtained using attacker spleen cells from individual *Rfv-1*$^{b/d}$ (low recovery incidence) mice [(B10.A × A.BY)F_1, (B10.A(5R) × A.BY)F_1, and (B10.A(18R) × A.BY)F_1] tested against ^{51}Cr-labeled A13 target cells at various times after i.v. inoculation of FV (1,500 or 15,000 FFU (B10.A × A.BY)F_1 stock). Greater than 5% cytotoxicity was considered significant.

against viral-induced cell surface antigens and not against normal spleen cell antigens.

Comparison of CMC with Antibody and C′-Mediated Cytotoxicity. Using a series of mouse leukemia cell lines, we compared the specificity of the CMC assay to the specificity of cytotoxicity mediated by antibody and C′. Antiserum and attacker spleen cells used were obtained from (C57BL/10 × A.BY)F_1, (B10.A × A.BY)F_1, and (B10.A × A/WySn)F_1 mice 20–40 days after inoculation with FV. The results (Table II) indicated a striking difference in the specificity of lysis with these two techniques. All leukemia virus-induced cell lines were lysed by antibody and C′. However, with one exception (BC1), only the leukemia virus-induced lines possessing *H-2*^{b} specificities were killed in the CMC assay system. BC1, the only *H-2*$^{d/d}$ line to show any lysis, was always lysed to a lesser extent than the *H-2*$^{b/b}$ lines, and in many cases was negative with attackers that gave strongly positive lysis using A13 target cells. Among the positive *H-2*$^{b/b}$ lines there was some consistent variation in the amount of CMC observed, which suggested that some differences in cell surface antigenicity might be present. For

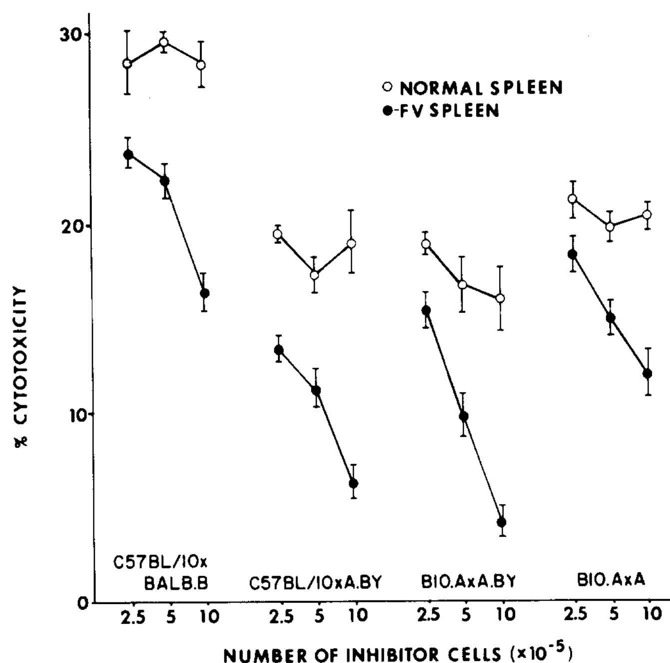

FIG. 3. Quantitative inhibition of CMC by normal and FV-induced 8–10 day leukemic spleen cells: (C57BL/10 × BALB.B)F_1 (H-$2^{b/b}$); (C57BL/10 × A.BY)F_1 (H-$2^{b/b}$); (B10.A × A.BY)F_1 (H-$2^{a/b}$); (B10.A × A/WySn)F_1 (H-$2^{a/a}$); (C57BL/10 × A.BY)F_1 anti-FV attacker spleen cells and ^{51}Cr-labeled A13 target cells were used. Values shown are the means ± standard error of the mean from three experiments.

example, relatively poor lysis was always obtained using RBL-5.[4]

Many of the cell lines were tested with the CMC inhibition assay. A13, 58, and BB4 showed strong inhibition of the lysis of ^{51}Cr A13 cells. RBL-5 showed weaker inhibition, and none of the H-$2^{d/d}$ lines, including BC1, showed inhibition. These results confirm the findings of the direct CMC tests and indicate that even though H-$2^{d/d}$ tumor cells have virus-induced surface antigens detectable by antibody and C′, they do not possess the appropriate viral-induced antigens for detection by direct CMC or inhibition of CMC.

One surprising finding in these experiments was the inability of (B10.A × A/WySn)F_1 (H-$2^{a/a}$) anti-FV attacker cells to lyse A13 or any of the other H-$2^{b/b}$ target cells lysed by congenic H-$2^{b/b}$ or H-$2^{a/b}$ anti-FV attacker cells. H-$2^{a/a}$ attackers were consistently able to lyse only line 58 target cells (H-$2^{b/h\text{-}35g}$). This observation could possibly be explained by a requirement for partial H-2 identity between target and attacker in this virus-specific lysis, as has been reported in the lymphocytic choriomeningitis (LCM) virus system (21).

Effect of Humoral Antibody on CMC. It was of major interest to study the effect of humoral antibody on the CMC assay. Normal mouse serum, anti-H-2^b

[4] In a similar CMC assay system using C57BL spleen cells from MSV inoculated mice (29), the opposite situation was observed as greater lysis was seen with RBL-5 targets than with A13. R. Herberman, personal communication.

TABLE II

Comparison of Antibody Plus Complement-Mediated Cytotoxicity with CMC for a Series of Lymphoma Cell Lines

Tumor	Strain	*H-2* type	Virus*	Antibody and C′-mediated cytotoxicity‡	Cell-mediated cytotoxicity§						Inhibition of CMC‖
					$H\text{-}2^{b/b}$ anti-FV attacker cells		$H\text{-}2^{a/b}$ anti-FV attacker cells		$H\text{-}2^{a/a}$ anti-FV attacker cells		
				%C	%C	%C vs. A13	%C	%C vs. A13	%C	%C vs. 58	
A13	BALB.B	b/b	B/T-L	50	56.0	—	15.2	—	−0.6	15.9	+
BB4	BALB.B	b/d	B/T-L	55	47.0	56.0	15.2	15.2	−3.1	15.9	+
58	(B10.A(4R) × A.BY)F_1	b/h-35g	FV	41	29.4	56.0	10.0	15.2	15.9	—	+
RBL-5	C57BL/6	b/b	RLV	NT	33.1	56.0	10.0	15.2	−7.9	10.1	+
FBL-3	C57BL/6	b/b	FV	40	39.6	56.0	15.7	15.2	0.2	15.9	+
EL4	C57BL/6	b/b	—	5	−0.4	56.0	2.6	15.2	0.1	10.1	−
BC1	BALB/c	d/d	B/T-L	54	12.1	56.0	5.8	13.2	3.0	15.9	−
BC2	BALB/c	d/d	B/T-L	68	3.3	56.0	3.7	13.2	3.0	15.9	−
BC-3A	BALB/c	d/d	B/T-L	80	3.7	19.3	NT	—	NT	—	−
LSTRA	BALB/c	d/d	MolLV	44	1.2	16.1	NT	—	NT	—	−
T2	BALB/c	d/d	FV	68	3.1	39.5	0.9	12.0	0.8	10.8	−
T4	BALB/c	d/d	FV	65	2.3	39.5	−1.1	12.0	1.6	10.8	−
T5	BALB/c	d/d	FV	53	1.6	39.5	−0.7	12.0	−2.1	10.8	−
HFL/d-sc	BALB/c	d/d	FV	46	1.3	39.5	−7.8	12.0	−9.4	10.8	−

* Denotes virus believed to have induced the primary neoplasm from which each line was derived.

‡ ^{51}Cr release assay with (C57BL/10 × A.BY)F_1 anti-FV serum and rabbit C′ was used as described (12). Cells were also examined in an indirect fluorescence assay using the same anti-FV serum followed by fluorescein-conjugated rabbit antimouse Ig serum. All tumor lines tested were positive except EL4.

§ CMC was assayed using spleen cell attackers from mice that had recovered from FV-induced splenomegaly. $H\text{-}2^{b/b}$ anti-FV attacker cells were from (C57BL/10 × A.BY)F_1 mice given 15,000 FFU FV i.v.; $H\text{-}2^{a/b}$ anti-FV attacker cells were from (B10.A × A.BY)F_1 mice given 1,500 FFU FV i.v.; $H\text{-}2^{a/a}$ anti-FV attacker cells were from (B10.A × A/WySn)F_1 mice given 150 FFU FV i.v. Spleens were used 11–30 days after FV inoculation.

‖ Inhibition of CMC by $H\text{-}2^{b/b}$ anti-FV spleen cells against A13 targets was tested using inhibitor:target ratios of: 5, 10, and 20. The criteria for positive inhibition were the same as described by Herberman et al. (31).

serum, and serum from mice that had recovered from FV-induced splenomegaly were added to the CMC assay system. In addition to the usual normal spleen and anti-FV spleen attackers, allogeneic attacker cells ($H\text{-}2^d$ anti-$H\text{-}2^b$) were obtained by immunizing with a non-FV tumor line (EL4). The results are presented in Table III. As has been previously reported using other target cells (22, 23) the anti-$H\text{-}2^b$ serum was effective in inhibition of the anti-EL4 (anti-$H\text{-}2^b$) attackers. The anti-FV sera had no such inhibitory effect on these attacker cells. When (C57BL/10 × A.BY)F_1 anti-FV attacker cells were used, no inhibition was observed (Table III). Several experiments were carried out using anti-FV sera, some with titers equal to that of the anti-$H\text{-}2^b$ serum previously used as a successful inhibitor of anti-$H\text{-}2^b$ attackers; however, in no case was specific inhibition of anti-FV attacker spleen cells seen. Preliminary experiments using FV suspensions (15,000 FFU/well) and in vitro prepared FV-antibody complexes also failed to inhibit CMC (data not shown).

ADNCC was demonstrated with the anti-$H\text{-}2^b$ serum, but not with anti-FV sera (Table III). The reason for this discrepancy is not known.

Characterization of Attacker Spleen Cells. To clarify the roles of different types of spleen cells as affectors in this CMC system, experiments with anti-θ and anti-Ia8 were carried out. The Ia8 antigen has been found in large quantity

TABLE III
Effect of Various Sera on Cell-Mediated Cytotoxicity Using A13 Target Cells

Serum*	Attacker spleen cells‡				
	Normal $H\text{-}2^{d/d}$	$H\text{-}2^{d/d}$ anti-EL4	Normal $H\text{-}2^{b/b}$	$H\text{-}2^{b/b}$ anti-FV	$H\text{-}2^{b/b}$ anti-FV
Diluent only	4.7 ± 0.8	77.9 ± 0.4	−1.8 ± 0.5	24.4 ± 1.6	38.0 ± 1.3
Normal H-$2^{b/b}$ serum	0.9 ± 0.5	68.8 ± 2.6	−0.6 ± 0.5	22.7 ± 1.0	33.5 ± 1.3
Anti-H-2^{b} serum	12.0 ± 0.8§	46.2 ± 1.5§	0.1 ± 0.6	18.8 ± 1.5	NT
H-$2^{b/b}$ anti-FV serum	0.6 ± 0.7	71.5 ± 0.9	1.8 ± 1.0	19.7 ± 0.5	37.1 ± 1.7
H-$2^{a/b}$ anti-FV serum	NT	NT	NT	NT	34.3 ± 0.8

* Using antibody and complement-mediated cytotoxicity and A13 target cells, the anti-H-2^{b} serum had a titer of 512, the H-$2^{b/b}$ anti-FV serum had a titer of 128, and the H-$2^{a/b}$ anti-FV serum had a titer of 512.

‡ $H\text{-}2^{d/d}$ mice used were (B10.D2 × BALB/c)F_1. Anti-EL4 spleen cells were obtained 10–14 days after i.p. injection of 1 × 10^6 viable EL4 cells. $H\text{-}2^{b/b}$ mice were (C57BL/10 × A.BY)F_1. Values presented are percent CMC ± SE of the mean. 0.050 ml of a one:five serum dilution was added to each well. The final well volume was 0.175 ml and contained 2 × 10^6 spleen cells plus 1 × 10^4 ^{51}Cr-labeled A13 target cells.

§ *P* value <0.01 compared to the same attackers in the presence of normal mouse serum.

only on B lymphocytes, and spleen cells killed by this serum in the presence of C′ can be assumed to be B cells (20). Anti-θ serum, on the other hand, kills only T lymphocytes. As shown in Table IV, exposure of attacker spleen cells to anti-θ serum plus C′ consistently abolished all CMC activity, whereas anti-Ia8 plus C′ or normal mouse serum plus C′ had no effect on CMC. This strongly implies that T lymphocytes are necessary for the CMC observed, and that B lymphocytes are not necessary.

Adherent cells or macrophages have been implicated in tumor immunity (24). To see if these cells were necessary for lysis in this system, glass-adherent spleen cells were removed from attacker populations, and the nonadherent cells were tested in the CMC assay. The results (Table V) show that nonadherent cells are equal to unfractionated populations in CMC activity.

The duration of incubation may be a critical factor influencing the results obtained in different CMC assay systems. Short (3–6 h) ^{51}Cr release assays detect only the original effector population in the organ tested, whereas longer (24–48 h) microcytotoxicity assays allow in vitro proliferation and differentiation of effector cells to occur (5, 25). Different results have been obtained in the MSV system using these two assays (26). Since the incubation period of our CMC assay was of intermediate duration (19 h), we decided to determine the contribution of cell division by the attacker population to the CMC activity observed. The effect of 2,000 R irradiation of attacker spleen cells was studied. This amount of irradiation inhibits cell division without killing cells. As seen in Table V, this treatment lowered the CMC activity, but did not abolish it completely. The results imply that there are effector cells present in the attacker spleens at the time the spleen is removed, however, some additional mitosis in vitro apparently takes place which further amplifies the CMC activity.

TABLE IV
CMC Activity after Treatment of Attacker Spleen Cells with Anti-θ or Anti-Ia8 Serum Plus Complement

Experiment	Treatment of attacker spleen cells		
	NMS* + C′	Anti-θ + C′	Anti-Ia8 + C′
1	30.2 ± 0.8‡	−0.6 ± 0.1	29.3 ± 1.0
2	17.4 ± 0.4	−0.9 ± 0.5	15.6 ± 0.6
3	10.0 ± 1.0	−0.4 ± 0.8	8.6 ± 1.4
4	37.9 ± 1.5	1.0 ± 0.3	NT§
5	27.2 ± 0.4	1.2 ± 0.4	NT

* Normal mouse serum.
‡ Values presented are percent CMC ± SE of the mean obtained in a CMC assay using A13 target cells and (C57BL/10 × A.BY)F_1 anti-FV attacker spleen cells.
§ NT, not tested.

Discussion

Spontaneous recovery from FV-induced leukemia has been shown to be influenced by many variables including virus strain (27), initial virus dose (9, 11), and several host genes (8–11). The present experiments provide evidence for the presence of specific cytotoxic effector cells in the spleens of mice during and shortly after recovery from FV-induced splenomegaly. Mice of both *Rfv-1*$^{b/b}$ (high recovery incidence) and *Rfv-1*$^{d/b}$ (low recovery incidence) genotypes were able to produce effector cells in association with recovery from splenomegaly. Therefore this gene does not appear to function via an absolute control over production of these cells. Among mice of either genotype who had recently recovered from splenomegaly, over 85% were positive in the CMC assay; only 2 of 26 mice with significantly enlarged spleens were positive. In all strains tested there was a very strong statistical correlation between presence of specific cytotoxic effector cells in the spleen and recent recovery from splenomegaly. Data from the previous paper indicated that no such correlation existed between presence of specific cytotoxic antibody and recovery from splenomegaly in these same mouse strains (12). The available evidence supports the hypothesis that the effector cells detected in the in vitro CMC assay may play an important role in the process of recovery from splenomegaly in vivo.

It was consistently observed that spleen cells from some individual mice who had apparently recovered from splenomegaly did not produce in vitro lysis of A13 target cells. It is possible that this could be due to the inhibition of the CMC assay system by residual leukemia cells present in these spleens. This could also explain our failure to see a transient peak of CMC activity in the spleens of progressor animals as has been found in the MSV system (28, 29). Although spleen palpation has proven to be a relatively accurate measure of recovery from splenomegaly when weekly examinations are made over the course of several months, a single palpation is a crude index of disease state at any given time, and the presence of a small population of persistent leukemia cells would be extremely difficult to rule out.

TABLE V
Effects of Removal of Glass Adherent Cells or 2,000 R Irradiation on Attacker Spleen Cell Populations

Experiment	Treatment of attacker spleen cells		
	None	Glass adherent cells removed	2,000 R
1	24.6 ± 0.8	22.2 ± 0.2	NT‡
2	15.4 ± 0.6	15.1 ± 0.5	NT
3	26.3 ± 1.3	20.4 ± 1.2	NT
4	38.2 ± 1.5	NT	9.4 ± 0.9
5	22.1 ± 0.6	NT	12.1 ± 1.0
6	60.5 ± 1.3	NT	26.9 ± 1.7

* Values presented are percent CMC ± SE of the mean obtained in a CMC assay using A13 target cells and (C57BL/10 × A.BY)F_1 anti-FV attacker spleen cells.
‡ NT, not tested.

Inhibition CMC tests with normal spleen cells, FV infected spleen cells, and FV itself indicated the specificity of immune lysis of A13 target cells was for FV-induced cellular antigens. However, examination of a series of leukemia cell lines as inhibitors or targets in the CMC assay suggested that the viral-induced cell membrane antigens detected in CMC were different from the antigens detected by techniques using humoral antibodies. Leukemia lines of $H\text{-}2^{b/b}$ and $H\text{-}2^{d/d}$ types, induced by FV, Moloney leukemia virus, Rauscher leukemia virus, and B/T-L virus, all reacted with antibodies from mice that had recovered from FV leukemia. However, among these serologically positive cell lines, only those containing $H\text{-}2^{b}$ antigens were positive in the CMC assay using virus immune attackers. One chemically-induced $H\text{-}2^{b/b}$ leukemia (EL4) did not react in either the antibody or cell-mediated virus-specific assays. All cell lines listed in Table II could be lysed by allogeneic attacker cells immunized to the *H-2* specificities present on the particular leukemia cell line. BC1 was the only $H\text{-}2^{d/d}$ cell line to show any sign of cell-mediated lysis by anti-FV attacker cells. This line was negative in the inhibition of lysis of A13 cells; however, in some assays with certain highly reactive $H\text{-}2^{b/b}$ attacker cells a low level of significant direct CMC was observed. Since it has previously been shown with LCM virus that virus-specific lysis of infected target cells by immune lymphocytes requires partial identity between target and attacker cells at the *H-2* locus (21), we also tested all our leukemia lines in CMC using immune attacker cells from $H\text{-}2^{a/b}$ mice, which have *d* allele specificities in the *D* region of the *H-2* complex. These attacker cells lysed the virus-antigen-positive $H\text{-}2^{b/b}$ cells, but failed to lyse the $H\text{-}2^{d/d}$ cells. This observation would appear to rule out the possibility that the lack of virus-specific lysis of $H\text{-}2^{d/d}$ cell lines was due to a requirement for partial identity at *H-2* similar to that reported for the LCM virus system. However, recent data by Shearer et al. (30) indicate that the requirement for *H-2* identity may be restricted to a specific region of the *H-2* complex. Identity for the *H-2D* region in the case of $H\text{-}2^{a/b}$ attackers and $H\text{-}2^{d/d}$ targets may not be sufficient for lysis on

this basis. (B10.D2 × A.BY)F_1 ($H\text{-}2^{d/b}$) attacker cells will have to be tested to completely rule out this possibility.

One unexpected finding could possibly indicate that a requirement for *H-2* identity may operate to some extent in the FV system. $H\text{-}2^{a/a}$ anti-FV spleen cells were able to lyse only line 58 target cells ($H\text{-}2^{b/h-35g}$), which have *H-2K* region specificities identical with $H\text{-}2^{a}$ (Table II). In contrast, $H\text{-}2^{b/b}$ cell lines, which were lysed by $H\text{-}2^{b/b}$ and $H\text{-}2^{a/b}$ attackers, were not lysed by $H\text{-}2^{a/a}$ attacker cells. Due to rarity of regression of splenomegaly in $H\text{-}2^{a/a}$ mice, $H\text{-}2^{a/a}$ anti-FV attacker spleen cells have not been studied by inhibition with normal and FV leukemic spleen cells to determine whether their lysis of line 58 cells is specific for virus-induced cell surface antigens. It is theoretically possible that some nonviral related antigens are involved in this lysis.

On the other hand, one additional piece of data should be stressed concerning the possible role of an *H-2* identity requirement in our system. $H\text{-}2^{a/a}$ FV leukemic spleen cells were able to inhibit lysis of $H\text{-}2^{b/b}$ anti-FV attacker cells (Fig. 3). In this situation, the inhibitor leukemia cells shared no partial *H-2* identity with either targets or attackers, and the inhibition appeared to be specific for virus-induced cell surface antigens only. A similar result has been obtained using immunization in vitro with trinitrophenyl (TNP)-modified autologous spleen cells (30). In some cases, lysis was independent of *H-2* compatibilities and appeared to be hapten-specific only.

The results of CMC inhibition experiments using various sera may be pertinent to understanding of the specificities toward which the CMC activity is directed. $H\text{-}2^{d/d}$ spleen cells immunized against virus-negative $H\text{-}2^{b/b}$ EL4 leukemia cells can lyse both EL4 and A13 target cells. The major antigens recognized are $H\text{-}2^{b}$ specificities, and the lysis can be inhibited by anti-$H\text{-}2^{b}$ serum produced in $H\text{-}2^{d/d}$ mice. This confirms the observation that antibodies to the appropriate determinants can inhibit CMC in a ^{51}Cr release assay (22, 23). Our efforts to obtain a similar specific inhibition using immune serum and attacker cells from mice, after recovery from FV leukemia, were unsuccessful. The explanations for both the lack of inhibition of virus-specific CMC by anti-FV sera and the lack of lysis of virus antigen-positive $H\text{-}2^{d/d}$ leukemia cells are unclear. Our data support the idea that the specificities detected by CMC and antibody and C′ cytotoxicity are not identical. A similar conclusion was reached by two groups using the MSV system (28, 31). Herberman et al. (31) hypothesized that the antigens detected in CMC after injection of MSV into C57BL/6 mice could be related to expression of an endogenous C-type virus, rather than to exogenous murine sarcoma virus. Alternatively, it is possible that the antigens detected in CMC are in fact directly induced by the exogenous murine viruses. The variation observed in the cell-mediated lysis of various leukemia target cell lines could merely be a reflection of quantitative differences of expression of the pertinent cell surface antigens.

Several different effector mechanisms of lysis could be ongoing simultaneously when unfractionated spleen cells are mixed with tumor target cells (5–7). Using specific antisera capable of selectively killing T or B spleen lymphocytes we investigated the nature of the effector cells demonstrated in this CMC system. The results indicated that the effectors are T lymphocytes, as all CMC activity was abolished when these cells were killed. Using anti-Ia8 serum and

complement to kill the B lymphocytes in the attacker spleen population had absolutely no effect on the CMC observed. ADNCC, a mechanism of lysis believed to be mediated by non-T lymphocytes (5), was not seen in this system using anti-FV serum and normal spleen cells, whereas ADNCC was easily demonstrated with an anti-H-2^b serum of equal titer. In addition, removal of glass-adherent cells from the spleen population did not abolish or reduce the CMC activity observed. All these experiments indicate that the effector cells from anti-FV spleens are T lymphocytes which function in vitro without assistance or inhibition by B lymphocytes or macrophages.

In tumor immunology most authors, including ourselves, rely heavily on correlation of in vitro findings with in vivo observations. However, such results must be confirmed by more direct experimentation involving in vivo transfer of fractionated effector cell populations, antibodies, or inhibitory factors to tumor-bearing animals. This has been carried out to some extent in the MSV system, and the results suggest T lymphocytes are effective against tumor challenge in vivo (32, 33). In another laboratory, passive transfer of antiserum has also proven effective against tumor challenge (34). However, passive transfer of serum or cells before or just after tumor challenge may not be equivalent to rejection of an established tumor. In the case of FV leukemia, passively transferred antiserum is effective against small numbers of early (1–3 day) FV spleen colonies, whereas such antibody appears to have little effect on the recovery from leukemic splenomegaly which can be seen in this system (12).

The role of nonspecific systemic or local effector mechanisms in recovery from FV leukemia has not been examined in this study. It is evident that such mechanisms play an important role in control of virus infection (35) and tumor growth (36, 37) in other systems, and most certainly are a significant factor in murine leukemia as well (38). Understanding the complex interactions and host genetic controls of these mechanisms should be a major goal in the field of tumor biology.

Summary

Congenic mouse strains differing only at genes within the *H-2* complex were found to have virus-specific cytotoxic effector cells in their spleens during or after recovery from Friend leukemia virus-induced splenomegaly. These effector cells were θ-positive T lymphocytes which functioned in vitro without help or inhibition by B lymphocytes or glass-adherent cells. The antigenic specificities recognized by the effector cells were viral-induced cellular antigens apparently different from those identified by serological techniques.

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