STUDIES OF CLONED FRIEND ERYTHROLEUKEMIA TUMOR CELLS

Modulation of the Tumor-specific Cytolytic T Lymphocyte Response by Infectious Friend Virus Production In Vitro*

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The Friend virus complex (Friend LV)[1] (1, 2) induces erythroleukemias in mice. The replication-defective component of Friend LV, spleen focus-forming virus (SFFV), includes in its genome a gene whose expression transforms infected erythroblastic cells. SFFV depends on coinfection with a competent helper virus, Friend murine leukemia virus (FMuLV), for a portion of its replicative cycle. Each coinfecting virus expresses gene products, some of which become located on the host cell surface and function as tumor-specific antigens.

Cultured lines of cells transformed in vivo by leukemogenic viruses have served widely in studies of the immune response to these cell surface antigens. Our laboratory has studied the *H-2*-restricted cytolytic T lymphocyte (CTL) component of this response using a series of Friend LV-induced erythroleukemia cell lines derived from mice of strains congenic with BALB/c but differing with respect to their *H-2* haplotypes (3). Although this CTL response has viral specificity, the viral proteins bearing the specificities have not been identified with certainty. However, it seems clear that both the SFFV and FMuLV genomes code for molecules with the capacity to generate a CTL response under appropriate conditions (4, 5).

These studies represent a preliminary analysis of clones of the previously uncloned line of erythroleukemia cells, HFL/b, induced in a BALB.B mouse by Friend LV (6). Studies of viral antigen expression by virus-producer and -nonproducer clones of this line revealed no major serologically detectable differences, but virus-producer clones were markedly more sensitive to lysis by syngeneic Friend LV-specific CTL than

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[1] *Abbreviations used in this paper:* BI_{50}, binding index, defined as the reciprocal serum dilution giving 50% binding of protein A; CI_{50}, cytotoxic index, defined as the lymphocyte:target cell ratio giving 50% lysis of 10^4 target cells; CTL, cytolytic T lymphocyte; FBS, fetal bovine serum; FMR, cell surface antigen induced by Friend, Moloney, and Rauscher leukemia viruses; FMuLV, Friend murine leukemia virus; Friend LV, Friend virus complex; LU, lytic unit; MEM, Eagle's minimal essential medium; MLC, allogeneic secondary mixed leukocyte cultures; MLTC, syngeneic mixed leukocyte-tumor cell cultures; PBS, phosphate-buffered saline, pH 7.2; PFU, plaque-forming units; RTase, reverse transcriptase; SFFU, spleen focus-forming units; SFFV, spleen focus-forming virus.

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nonproducer clones. However, virus-producer clones appeared to be less efficient than nonproducer clones as stimulators of CTL generation in syngeneic mixed leukocyte-tumor cell cultures (MLTC). Our results thus indicate that virus production or nonproduction by HFL/b tumor cells is an important variable with a complex influence on the Friend LV-specific CTL response.

Materials and Methods

Mice. Mice of the inbred congenic strains BALB.B ($H\text{-}2^b$) and BALB/c ($H\text{-}2^d$) were bred in our colony at the Albert Einstein College of Medicine, Bronx, N. Y.

Tumor Cells. HFL/b cells were derived from the spleen of a BALB.B mouse with erythroleukemia induced by Friend LV (6). RBL-5 cells came from a lymphoma induced in a C57BL/6 mouse ($H\text{-}2^b$) by Rauscher leukemia virus (LV) (7). LSTRA cells were induced in a BALB/c mouse by Moloney LV (8). B.GV cells were derived from a BALB.B Gross LV-induced leukemia (9). These four tumors were maintained as continuous suspension cell cultures in Eagle's minimal essential medium (MEM) supplemented with 20% fetal bovine serum (FBS). Each culture was passaged about every 4 d by transferring 10^6 cells into 5 ml fresh culture medium.

Cloning Procedure. HFL/b cells were cloned by limiting dilution. Cell suspensions containing one cell in 40 μl were distributed in 10-μl aliquots into 60-well tissue culture microplates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) and examined to identify wells containing a single cell. These wells were refed 4 d later, and the clones were allowed to proliferate. When sufficiently expanded, the clones were passaged every 4 d as suspension cultures. Plating efficiency for HFL/b cells was ~62%.

Virus Assays. Each culture to be assayed for virus production was initiated with 5×10^6 cells in 5 ml fresh tissue culture medium and incubated for 24 h at 37°C. Culture supernates were collected and centrifuged once at 600 *g* for 5 min and again at 1,500 *g* for 10 min, and then tested for the presence of virus particles with one or more of the following methods:

(*a*) *Assay for Reverse Transcriptase (RTase) Activity.* Each cell-free supernate was cleared by centrifugation at 5,900 *g* for 7 min. The virus particles were then pelleted by centrifugation at 108,000 *g* for 90 min and resuspended in 50 μl phosphate-buffered saline, pH 7.2 (PBS). The RTase activity of these viral preparations was measured with a modification (10) of the method of Scolnick and Parks (11).

(*b*) *Spleen Focus Assay.* The presence of Friend LV in the cell-free culture supernates was detected in vivo with the assay of Axelrad and Steeves (12), which measures SFFV in the presence of FMuLV. Supernate dilutions ranging from 1/1 to 1/1,000 (in PBS) were injected intravenously into the lateral tail vein of BALB/c mice in 1-ml vol. The mice were killed 9 d later, and the spleens were removed and fixed in Bouin's solution. Discrete macroscopic focal lesions on the surface of the spleen were counted, and virus titers were expressed in spleen focus-forming units (SFFU) per ml culture supernate.

(*c*) *XC Cell Line Plaque Assay.* A modification of the method of Rowe et al. (13) was used to measure the activity of helper FMuLV in cell-free culture supernates, as described recently (14). Serial dilutions of culture supernates were used to infect a clonal derivative of SC-1 mouse cells (obtained from Dr. P. O'Donnell, Memorial Sloan-Kettering Cancer Institute, New York), and the presence of FMuLV was revealed by syncytial plaque formation in the cultures after overlaying with rat XC cells. Virus titers were calculated in terms of plaque-forming units (PFU) per ml culture supernate.

(*d*) *Equilibrium Density Gradient Centrifugation of Labeled Virus Particles.* HFL/b cells were suspended in 15 ml complete culture medium at 10^6 cells per ml, and 200 μCi [5,6-^{3}H]uridine or [2-^{14}C]uridine (Amersham Corp., Arlington Heights, Ohio) was added. After a 6-h pulse at 37°C, the cells were centrifuged at 600 *g* and the cell pellet was resuspended in 10 ml fresh culture medium. The cells were further incubated for 16 h at 37°C, and the culture supernates were cleared as described above. 10 ml supernate was layered on a linear 20–60% sucrose gradient (7 ml) and centrifuged at 94,500 *g* for 3 h. The tube was punctured, and 3-drop fractions were collected from the bottom. The refractive index of each fraction was determined, and each fraction was counted differentially for β-radioactivity in a well-type liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Radioimmunoassay. Cell-surface antigens were detected with a radioimmunoassay using ^{125}I-labeled *Staphylococcus aureus* protein A, which binds to the Fc portion of immunoglobulin, according to the method of Dorval et al. (15). Purified protein A (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) was iodinated with Na[^{125}I] by the chloramine-T-coupling procedure of Hunter and Greenwood (16). Radiolabeled protein A was separated from free iodine by Sephadex G-25 (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) chromatography and stored at −70°C. The radioimmunoassay was initiated by incubating 10^6 cells in 100 μl MEM with 10 μl antiserum (diluted serially in MEM) in 6- × 50-mm glass test tubes for 60 min at 4°C. After three washes in 2 ml MEM, the cells were resuspended in 100 μl MEM and incubated with 2 × 10^5 counts of ^{125}I-protein A for 30 min at 4°C. The cells were washed three times, and the final cell pellet was counted for radioactivity in a well-type gamma (Searle Radiographics Inc., Des Plaines, Ill.) counter. The relative degree of ^{125}I-labeled protein A binding was calculated according to the formula:

$$\text{Percent binding} = \frac{\text{EB} - \text{BB}}{\text{MB} - \text{BB}} \times 100,$$

where EB is the experimental binding; BB, the background binding in the absence of antiserum; and MB, the maximum binding obtained with a positive serum under saturating conditions. The absence of Fc receptors on HFL/b cells was confirmed by incubation of the cells in normal mouse serum or in irrelevant antisera; in both cases ^{125}I binding was equivalent to background binding in the absence of serum.

Antisera. Mouse anti-H-2.2 serum (anti-$H\text{-}2D^b$ serum) was prepared in our laboratory by hyperimmunizing (A × HTI)F_1 mice with EL4 ($H\text{-}2^b$) leukemia cells. Mouse anti-HFL/b serum was also prepared in our laboratory by repeated immunization of BALB.B mice with HFL/b cells. Goat antisera to Rauscher LV gp69/71, p30, and p12 antigens were obtained through the Office of Program Resources and Logistics, Viral Oncology Division, National Cancer Institute, Bethesda, Md. All antisera were heat-inactivated at 56°C for 30 min.

Lymphocyte Cultures. Friend LV-specific CTL were generated in culture from spleen cells of BALB.B mice primed intraperitoneally 20–30 d previously with 5 × 10^6 HFL/b cells inactivated by a 20-sec fixation in 0.15% glutaraldehyde (17). Syngeneic MLTC (18) were established in upright tissue culture flasks 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 × 10^7 spleen cells with 1 × 10^6 x-irradiated (5,000 rad) syngeneic HFL/b cells. Cultures were harvested after incubation for 6–7 d and were assayed for cytolytic activity with the ^{51}Cr-release cytotoxicity assay.

Secondary MLTC were also established as microcultures by incubating 5.0 × 10^6 HFL/b-immune BALB.B spleen cells with various numbers of x-irradiated (5,000 rad) or glutaraldehyde-fixed HFL/b cells in 2 ml MLTC medium. Glutaraldehyde fixation was performed as described by Bubbers and Henney (17), except that after fixation, the cells were incubated at 0°C for 24 h in serum-free MEM.

Allogeneic secondary mixed leukocyte cultures (MLC) were established to generate CTL specific for $H\text{-}2^b$ antigens. BALB/c ($H\text{-}2^d$) mice were immunized with two intraperitoneal injections of 3 × 10^7 BALB.B ($H\text{-}2^b$) spleen cells, and secondary MLC were established by mixing 2.5 × 10^7 $H\text{-}2^b$-immune BALB/c spleen cells with 2.5 × 10^7 x-irradiated (1,000 rad) BALB.B spleen cells in 20 ml culture medium.

^{51}Cr-release Cytotoxicity Assay. Cell-mediated cytolytic activity was detected by incubating 10^4 ^{51}Cr-labeled target cells with lymphocytes harvested from MLTC or MLC at various lymphocyte:target cell ratios, in a final vol of 0.2 ml in round-bottom multi-well plates according to the assay originally described by Brunner et al. (19). After incubation at 37°C for 6 or 15 h, the supernates were harvested and measured for radioactivity in a Searle well-type gamma counter. The percent specific ^{51}Cr release was calculated for each lymphocyte:target cell ratio according to the formula:

$$\text{Percent release} = \frac{\text{ER} - \text{SR}}{\text{MR} - \text{SR}} \times 100,$$

where ER was the experimental ^{51}Cr release; SR, the spontaneous ^{51}Cr release detected after incubation of the target cells in culture medium alone; and MR, the maximum ^{51}Cr release obtained after incubation of the target cells in 1 N HCl for the duration of the assay. Spontaneous release (SR, see above) values varied between 8 and 20% of total incorporated label. Dose-response curves were established for each lymphocyte population assayed by plotting specific cytolytic activity versus the $\log_{10}$ of the number of viable effector cells. The cytotoxic index (CI_{50}) of each lymphocyte population was defined as the lymphocyte-to-target cell ratio giving 50% lysis of 10^4 target cells.

The number of cytotoxic cells generated in micro-MLTC was quantitated in terms of lytic units (LU). 1 LU was defined in our HFL/b system as the number of viable MLTC cells necessary to lyse 20% of 10^4 ^{51}Cr-labeled HFL/b clone B-2 target cells during an incubation period of 6 h. This value was calculated graphically from the dose-response curves of cytolytic activity obtained with each micro-MLTC (cf. Fig. 5). Results were expressed in terms of LU per 10^6 MLTC cells and of total LU recovered per microculture.

Cold Target Cell Inhibition Assay. CTL specificity for target antigens was analyzed by adding unlabeled (cold) competitor target cells to the ^{51}Cr-release cytotoxicity assay. Variable numbers of cold target cells were mixed in multi-well plates with 10^4 ^{51}Cr-labeled target cells in 0.1 ml culture medium; 3×10^5 effector lymphocytes were subsequently added in 0.1 ml culture medium to each well, and the plates were incubated for 6 h at 37°C. The percent inhibition of cytotoxicity was calculated according to the formula:

$$\text{Percent inhibition} = \frac{\text{Cont} - \text{Exp}}{\text{Cont}} \times 100,$$

where "Cont" was the specific cytotoxic activity detected in wells containing no cold target cells and "Exp" was the experimental cytotoxicity values obtained from wells containing cold target cells, effector lymphocytes, and ^{51}Cr-labeled target cells.

Results

Heterogeneity among Clones of HFL/b Cells. The HFL/b tumor cell line originated from the spleen of a Friend LV-infected BALB.B mouse and was adapted to growth as a continuous line in culture, without cloning (6). HFL/b cells revived from frozen storage at the 65th passage were cloned after 7 further passages, when they were producing relatively large amounts of infectious Friend LV. 17 clones were obtained, all of which produced virus particles as indicated by release of sedimentable RTase activity. This enzyme has been shown to be incorporated into oncornavirus particles (20). Wide variations in RTase activity (471–19,532 cpm) were observed among the 17 clones (data not shown), and 3 clones (i.e., clones B, C, and L) were selected for recloning. Clone B showed the highest index of virus production and clones C and L, the lowest.

Two subclones were obtained from clone B, 15 from clone C, and 19 from clone L. When culture supernates were assayed in vivo for the induction of spleen foci by the SFFV component of Friend LV (Fig. 1), subclones B-1 and B-2, as well as the initial clone B, proved to be high virus producers (titers $\geq$ 550 SFFU/ml). Clone C yielded a majority of subclones which produced moderate to large amounts of infectious SFFV; however, two of the subclones, C-4 and C-10, produced very small amounts of virus (titers $\leq$ 60 SFFU/ml). Recloning of clone L yielded 19 subclones which were all moderate to high Friend LV producers; in fact, 12 of these subclones had titers >700 SFFU/ml (Fig. 1). These studies thus indicated that uncloned HFL/b cultures consisted of cells which were widely heterogeneous with respect to their capacity to produce infectious Friend LV.

Time-Course Studies of Shutdown of Friend LV Production. A transition from high virus

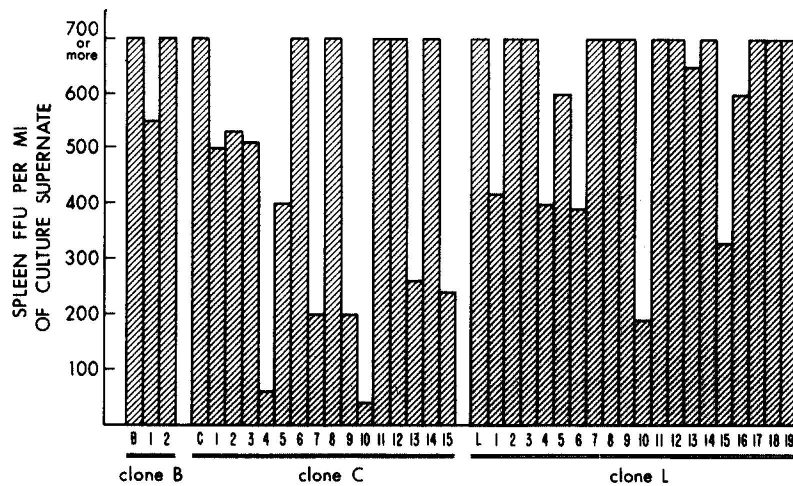

FIG. 1. Spleen focus-forming activity of HFL/b culture supernate.

producer to low producer or nonproducer status, occurring repeatably at about the same cumulative passage level, had been observed in the uncloned HFL/b cell line (10) and was again noted in the course of these experiments. Between passages 98 and 132 (i.e., an interval of ~162 d of continuous culture) virus titers of culture supernates fell from >800 to 30 SFFU/ml (Fig. 2). Parallel studies of Friend LV production by high producer clone B-2 and low producer clone C-4 (Fig. 2) showed that clone B-2 cells retained the ability to produce large amounts of infectious Friend LV throughout the same period in which uncloned HFL/b cells stopped producing infectious virus (i.e., passages 6–40 for clone B-2). Furthermore, clone B-2 cells have now maintained the ability to produce large amounts of infectious Friend LV for >18 mo in continuous culture. In contrast, clone C-4 cells produced very little infectious Friend LV; in fact, by passage 56, they had lost all capacity to produce detectable levels of virus (Fig. 2).

These data thus indicated that a repressive genetic mechanism occurring in a synchronous manner in each cell was apparently not the major cause of the transition to nonproducer status observed among uncloned HFL/b cells. Further cloning experiments (data not shown) indicated that asynchrony in the arrest of virus production was not an alternative explanation for our observations. HFL/b cells were cloned at passage 165, when they were producing very low amounts of virus. 13 clones were derived, all of which were low virus producers, with titers ranging from 0 to 30 SFFU/ml. Similarly, high producer clone B-2 was subcloned at passage 66, and the 28 clones obtained were all high producers of Friend LV (1,000–50,000 SFFU/ml).

These findings appear to eliminate the possibility that the transition from virus production to nonproduction by uncloned HFL/b cells was a genetically programmed event occurring quasi-synchronously in each cell. Rather, it seems likely that spontaneous shutdown of virus production occurs as a rare event in HFL/b cells, and that nonproducer cells then overgrow producer cells by virtue of a slight advantage in growth rate. However, no difference was detected in the growth of producer B-2 cells compared with nonproducer C-4 cells.

Surface Antigens of HFL/b Cloned Cells. HFL/b cells were assayed with specific antisera for a variety of cell surface antigens with the protein A radioimmunoassay. Table I

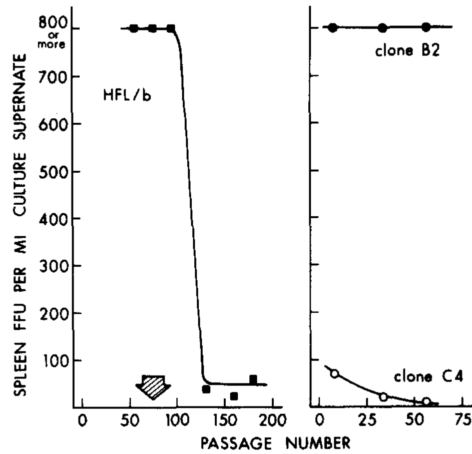

FIG. 2. Time-course of SFFV production by HFL/b cells. Uncloned HFL/b cells (■), as well as HFL/b subclones B-2 (●) and C-4 (○) were assayed in vivo for SFFV production at various times during their continuous culture. The arrow in (A) indicates passage No. 72, when HFL/b cells were first cloned. Passage No. 1 of subclones B-2 and C-4 coincided with passage No. 92 of the uncloned HFL/b cells.

TABLE I
Characterization of HFL/b Clones B-2 and C-4

HFL/b clone	Passage number	Infectious virus production		Cell surface antigens (BI_{50})*				
		SFFV	FMuLV	gp69/71	p30	p12	HFL/b‡	H-2.2
		SFFU/ml	*PFU/ml*					
B-2	61	2.2×10^4	1.8×10^3	70	<10	<10	18	10
C-4	66	0	0	65	<10	<10	45	10

* Determined with specific antisera by the protein A binding assay. Data are expressed as the reciprocal of the antiserum dilution yielding 50% binding index values (BI_{50}), calculated from the graphed data.
‡ Cell surface antigens recognized by BALB.B anti-HFL/b serum.

summarizes a typical experiment that showed that no major antigenic differences could be detected between virus-producing and -nonproducing cloned cells. Although clone B-2 cells were producing large amounts of both SFFV and helper FMuLV and clone C-4 cells were not, both clones bound comparable amounts of antibodies to viral envelope glycoprotein gp69/71. Both clones were also recognized by BALB.B anti-HFL/b serum. This serum contains antibodies to glycoprotein gp69/71 and to FMR antigen, a cell-surface antigen commonly present on cells infected with Friend, Moloney, or Rauscher LV. Viral *gag* proteins p30 and p12 were barely detectable on the surface of either type of cell. In addition, H-2.2 antigen ($H\text{-}2D^b$) was present in both clones in equivalent amounts.

The equivalence in viral antigen expression observed with cells from high producer clone B-2 and nonproducer clone C-4 could have resulted from the production by clone B-4 cells of a virus undetectable with our assays. Fig. 3 shows that this was probably not the case: attempts to label a virus produced by clone C-4 cells with [^{14}C]uridine failed to reveal a virus band after equilibrium density-gradient centrifu-

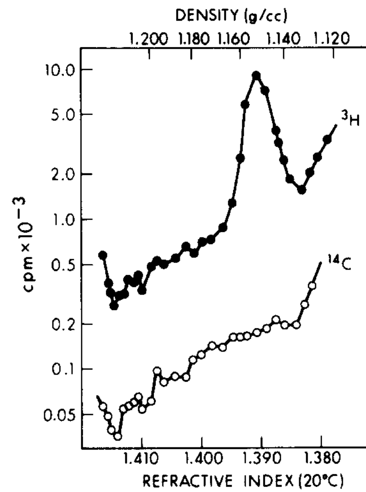

FIG. 3. Banding of labeled virus particles by equilibrium density-gradient centrifugation. After pulse-chase labeling of clone B-2 cells (●) with [^{3}H]uridine and of clone C-4 cells (○) with [^{14}C]uridine, 5 ml cell-free supernate from each culture was run on the same 20-60% sucrose linear gradient. After centrifugation, fractions were collected from the bottom of the tube and their refractive indices determined. The radioactivity of each fraction was counted differentially for ^{3}H and ^{14}C, and was plotted as a function of the refractive index.

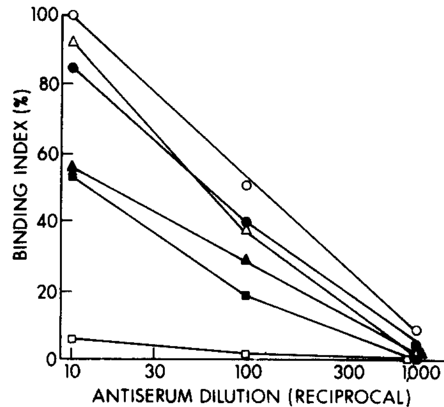

FIG. 4. Expression of gp69/71 on the surface of cloned HFL/b cells. Clone C and four subclones obtained from clone C were assayed with goat anti-Rauscher gp69/71 serum, using the protein A radioimmunoassay. ▲, clone C (high producer of Friend LV); △, subclone C-14 (high producer of Friend LV); ●, subclone C-4 (low producer of Friend LV); ○, subclone C-10 (high producer of Friend LV); ■, subclone C-15 (medium producer of Friend LV); □, normal BALB.B spleen cells.

gation in a 20-60% sucrose linear gradient. Similar labeling of clone B-2 cells with [^{3}H]uridine (as opposed to [^{14}C]uridine) gave rise to labeled particles that banded between 1.146 and 1.161 g/cm^{3} in the same sucrose gradient (Fig. 3).

That no major antigenic differences, as detected serologically, could be established between Friend LV-producing clones and -nonproducing clone C-4 was confirmed by the data shown in Fig. 4, in which levels of virus production by subclones derived from clone C showed no correlation with expression of viral gp69/71 on the cell surface. We concluded that, notwithstanding the inability to produce infectious

TABLE II
HFL/b Cells as Targets for CTL

^{51}Cr-labeled target cells	Passage number	Friend LV titer	Degree of lysis (CI_{50})	
			CTL anti-HFL/b	CTL anti-*H-2*^{b}
		SFFU/ml		
HLF/b (uncloned)	132	20	300:1	1.6:1
Clone B	55	>5 × 10^3	50:1	1.5:1
Clone B-1	40	4.5 × 10^3	100:1	1.5:1
Clone B-2	35	>5 × 10^3	15:1	1.0:1
Clone C	57	4.0 × 10^3	40:1	5.0:1
Clone C-4	40	20	>300:1	5.4:1
Clone C-10	40	3.0 × 10^3	90:1	3.0:1
Clone C-11	40	4.0 × 10^3	30:1	1.0:1
Clone C-14	40	3.5 × 10^3	50:1	1.4:1
Clone C-15	40	2.0 × 10^3	30:1	1.6:1

Cells from each clone were labeled with $Na_2[^{51}Cr]O_4$ and used as targets to CTL in a 15-h cytotoxicity assay. CTL were generated in syngeneic secondary MLTC against uncloned HFL/b tumor cells, and in allogeneic secondary MLC against *H-2*^{b} alloantigens.

Friend LV, clone C-4 cells retained the viral genetic material coding for the cell surface antigens examined.

Lysis of HFL/b Cloned Cells by CTL. In a further attempt to study the antigenic structure of HFL/b cells, various HFL/b clones were assayed for susceptibility to lysis mediated by syngeneic CTL generated against uncloned HFL/b cells. A long-term (15 h) ^{51}Cr-release cytotoxicity assay was used to detect all cytolytic reactions, including very weak reactions that would pass undetected with shorter assays. That these cytotoxic lymphocytes were indeed of the T cell lineage was indicated by their high degree of specificity for Friend LV-induced but not Gross LV-induced tumor cells (see below), by their susceptibility to the cytotoxicity of AKR anti-Thy-1.2 serum in the presence but not the absence of complement, and by the persistence of their activity after removal of macrophages and other phagocytic cells (data not shown).

The experiment summarized in Table II indicated that cells actively producing infectious Friend LV were more susceptible to lysis induced by HFL/b-specific CTL than low-producer cells; in fact, at least 20-fold fewer CTL were required to destroy 10^4 cells from clone B-2 than an equivalent number of cells from clone C-4 (i.e., CI_{50} values of 15:1 versus 300:1). However, when the same cells were used as targets for alloimmune CTL specific for *H-2*^{b} antigens, no major differences in susceptibility to lysis could be observed (Table II), thus indicating that none of the clones was inherently resistant to CTL-mediated lysis. A similar correlation between low virus production and relative resistance to tumor-specific CTL was detected with a series of low-producer and nonproducer clones (Table III), which contrasted with the susceptibility to lysis exhibited by high-producer clones B-2 and L.

Specificity of HFL/b-immune CTL. Fig. 5 shows that BALB.B CTL immune to HFL/b cells and generated in secondary MLTC were specific for *H-2*^{b} FMR-positive tumor target cells. These CTL killed *H-2*^{b} RBL-5 cells (induced by Rauscher LV)

TABLE III

Correlation between Friend LV Production by Cloned HFL/b Cells and Susceptibility to Lysis by HFL/b-specific CTL

^{51}Cr-labeled target cells	Passage Number	Friend LV titer	Lysis (percent specific ^{51}Cr release)*				CI_{50}
			100:1	10:1	1:1	0.1:1	
		SFFU/ml					
Clone B-2	61	22×10^3	85	64	56	39	0.7:1
Clone L	105	1×10^3	72	47	28	19	14:1
Clone C-4	66	0	30	17	9	5	>100:1
Clone HFL/b-3‡	2	35	36	11	0	0	>100:1
Clone HFL/b-4	2	7	43	17	4	0	>100:1
Clone HFL/b-5	2	0	37	15	4	4	>100:1
Clone HFL/b-6	2	0	38	8	0	0	>100:1
Clone HBL/b-7	2	0	12	2	0	0	>100:1
Clone HFL/b-8	2	0	35	13	5	4	>100:1
Clone HFL/b-9	2	0	38	8	4	0	>100:1
Clone HFL/b-10	2	25	26	6	0	0	>100:1
Clone HFL/b-11	2	0	22	12	0	0	>100:1
Clone HFL/b-13	2	19	35	18	3	0	>100:1

CTL were generated in syngeneic secondary MLTC against uncloned HFL/b tumor cells, and assayed for cytotoxicity on 10^4 ^{51}Cr-labeled cloned HFL/b cells in a 15-h assay.

* Multiple threefold lymphocyte dilutons were used; however, only a selection of lymphocyte:target cell ratios is represented, for the sake of brevity.

‡ Clones HFL/b-3 through HFL/b-13 were derived from uncloned HFL/b cultures at passage No. 165, when the latter were producing very low amounts of infectious Friend LV (cf. Fig. 2).

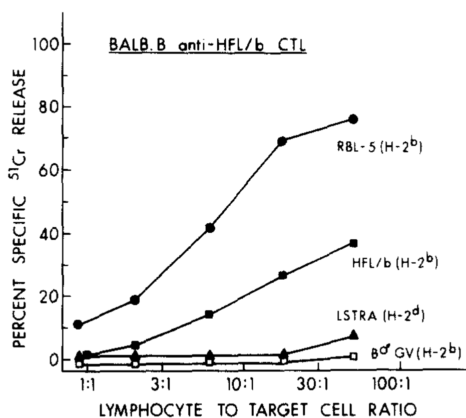

FIG. 5. Specificity of BALB.B CTL generated against uncloned HFL/b cells in secondary MLTC. CTL activity was detected in a 6-h cytotoxicity assay using four different ^{51}Cr-labeled tumor cell lines as targets: ●, RBL-5 (*H-2^b*); ■, HFL/b clone B-2 (*H-2^b*); ▲, LSTRA (*H-2^d*); □, B.GV (*H-2^b*).

and HFL/b clone B-2 cells in a 6-h cytotoxicity assay. Allogeneic (*H-2^d*) FMR-positive LSTRA cells (induced by Moloney LV) and syngeneic (*H-2^b*) FMR-negative B.GV cells (induced by Gross LV) were not attacked by the same CTL.

Other experiments were designed to study the finer specificity of HFL/b-immune CTL by cold target competitive inhibition of cytolysis. A 6-h ^{51}Cr-release cytotoxicity

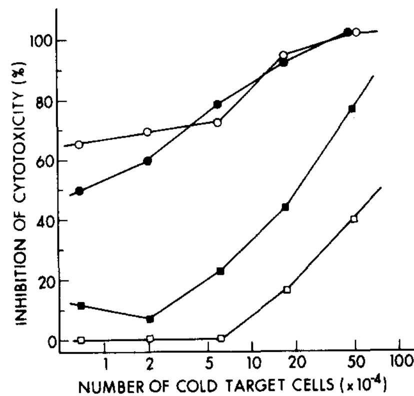

FIG. 6. Competitive inhibition of BALB.B anti-HFL/b CTL with unlabeled target cells. The specificity of CTL-mediated lysis of ^{51}Cr-labeled HFL/b clone B-2 target cells was studied in a 6-hr competition assay with the following unlabeled tumor cells: HFL/b clone B-2 cells (●), HFL/b clone C-4 cells (○), Gross LV-induced B.GV cells (■), and normal BALB.B spleen cells (□). Percent specific cytotoxicity in the absence of competitor cells was 23%.

assay was used to detect whatever fine differences in inhibitory capacities might exist among various tumor cells. When cells from Friend LV-producer clone B-2 and nonproducer clone C-4 were used as cold target cells to compete with ^{51}Cr-labeled clone B-2 target cells for lysis mediated by HFL/b-specific CTL, both types of cold target cells competed equally well (Fig. 6). However, when syngeneic Gross LV-induced B.GV cells were used as cold competitor cells, 20-fold more B.GV cells than clone C-4 or B-2 cells were required to achieve the same degree of inhibition; this difference increased to 40-fold when normal BALB.B spleen cells were used as cold competitors. A similar pattern of inhibition was observed when clone C-4 cells were used as ^{51}Cr-labeled targets, instead of cells from clone B-2 (data not shown). These results confirmed the indications of fine viral specificity among HFL/b-immune CTL, because Friend LV-induced tumor cells competed better than Gross LV-induced tumor cells. It was also apparent that cells from nonproducer clone C-4 expressed tumor antigens in amounts sufficient to block cytolysis as efficiently as cells from producer clone B-2.

Stimulating Capacity of HFL/b Cells in Secondary MLTC. In another series of experiments, we considered the ability of x-irradiated HFL/b cells to present tumor antigen in secondary MLTC, and thus to induce lymphocyte differentiation and generation of HFL/b-specific CTL. Preliminary screening experiments (Table IV) indicated that, contrary to expectations, Friend LV-producing clones were considerably less efficient in inducing CTL generation than nonproducer clones. Thus, at a fixed ratio of 10 responding spleen cells to 1 stimulating tumor cell, Friend LV-producer clones were at least 300 times less efficient in eliciting HFL/b-specific CTL than low-producer uncloned HFL/b cells or clone C-4 cells.

In view of the contrast between these results and those presented in the previous sections, the stimulating capacities of Friend LV-producing clone B-2 and nonproducing clone C-4 were studied in greater detail by means of a dose-response titration. For this purpose, microcultures were established by adding various numbers of stimulating tumor cells to 5×10^6 spleen cells from HFL/b-primed BALB.B mice. The number of CTL generated per micro-MLTC was quantitated in a short-term (6

TABLE IV

HFL/b Cells as Stimulators of CTL Differentiation in Secondary MLTC

Stimulating cells	Passage Number	Friend LV titer	CTL generation in secondary MLTC	
			Viable cells recovered	CI_{50}
		SFFU/ml	$\times 10^{-6}$	
HFL/b	132	20	1.9	1:1
Clone B	55	$>5 \times 10^3$	1.5	>300:1
Clone B-1	40	4.5×10^3	0.7	>300:1
Clone B-2	35	$>5 \times 10^3$	0.8	>300:1
Clone C	57	4.0×10^3	1.5	>300:1
Clone C-4	40	20	1.5	0.6:1
Clone C-10	40	3.0×10^3	1.9	>300:1
Clone C-11	40	4.0×10^3	2.0	300:1
Clone C-14	40	3.5×10^3	1.5	>300:1
Clone C-15	40	2.0×10^3	1.2	300:1

MLTC were established by mixing 5×10^6 HFL/b-immune BALB.B spleen cells with 0.5×10^6 x-irradiated (5,000 rad) stimulating tumor cells in 2 ml MLTC medium. After incubation for 7 d, each culture was tested for cytotoxicity on ^{51}Cr-labeled clone B-2 cells in a 15-h assay at various effector:target cell ratios.

h) ^{51}Cr-release cytotoxicity assay, to arrive at a minimum estimate of the CTL number. Results were expressed in terms of LU, as calculated from the dose-response cytotoxicity curves obtained for each individual MLTC. Table V summarizes a typical dose-response experiment in which it became apparent that Friend LV-producing cells (i.e., clone B-2 cells) could indeed stimulate CTL generation, but that this capacity was restricted to a narrow range, such that highest CTL values were obtained at a ratio of 83 responding spleen cells to 1 stimulating tumor cell (i.e., 6×10^4 tumor cells per culture), and no CTL generation could be detected at ratios of 10:1 (i.e., 5×10^5 tumor cells per culture) or higher. In contrast, cells producing low to undetectable levels of virus (i.e., uncloned HFL/b cells and clone C-4 cells) were capable of eliciting high levels of CTL generation over a considerably wider range, including ratios of 10:1 through 83:1 (i.e., from 5×10^5 to 6×10^4 stimulating cells per culture). Furthermore, a mixture of cells obtained by combining clones B-2 and C-4 in equal numbers showed a restricted range of stimulating capacity (Table V). CTL generation could no longer be detected with 5×10^5 B-2 + C-4 cells. These results thus indicated that Friend LV-producing cells could indeed stimulate CTL generation in MLTC, but their stimulating capacity was best observed with low numbers of stimulating cells per culture; in fact, they appeared to interfere with CTL generation at high doses.

Other experiments showed that the inhibition of CTL generation with 5.0×10^5 clone B-2 cells was probably a result of the production of infectious Friend LV by the x-irradiated stimulating cells during the initial phases of lymphocyte culture. Table VI shows that fixation of clone B-2 cells with 0.15% glutaraldehyde (instead of x-irradiation) diminished the inhibitory potential of clone B-2 cells, and relatively high

TABLE V

Dose-Response Studies of CTL Generation in Secondary MLTC

Stimulating cells (5,000 rad)	Stimulating cells per culture	CTL generation in MLTC (day 7)		
		Viable cells recovered	LU/10^6 cells	LU/culture
	$\times 10^{-6}$	$\times 10^{-6}$		
HFL/b (226)*	1.00	2.1	0	0
	0.50	1.6	4.0	6.4
	0.17	1.6	6.2	10.0
	0.06	2.1	4.0	8.4
	0.02	2.0	0.5	1.1
Clone B-2 (114)	1.00	1.2	0	0
	0.50	1.4	0	0
	0.17	1.9	1.2	2.3
	0.06	1.9	4.2	8.0
	0.02	2.1	1.3	2.7
Clone C-4 (118)	1.00	1.7	0	0
	0.50	2.0	1.7	3.4
	0.17	1.8	6.2	11.2
	0.06	2.0	7.1	14.2
	0.02	1.7	0.4	0.7
B-2 + C-4 (1:1 mix)	1.00	1.2	0	0
	0.50	1.8	0	0
	0.17	1.8	8.3	15.0
	0.06	2.1	12.5	26.2
	0.02	1.9	1.2	2.4

5×10^6 HFL/b-immune BALB.B spleen cells were mixed with variable numbers of x-irradiated stimulating tumor cells in 2 ml culture medium and incubated for 7 d. CTL generation was determined by assaying each culture for cytotoxicity with ^{51}Cr-labeled clone B-2 target cells at different effector:target cell ratios in a 6-h assay.

* Passage number is in parentheses. At this stage uncloned HFL/b cells were producing 30 SFFU/ml, clone C-4 cells were not producing detectable levels of Friend LV, and clone B-2 cells were producing 3,500 SFFU/ml.

numbers of CTL could be generated with 5×10^5 glutaraldehyde-fixed clone B-2 cells (i.e., a ratio of 10 responding spleen cells to 1 stimulating tumor cell), in contrast with the lack of response observed at this same ratio with x-irradiated clone B-2 cells. Finally, it was also observed that cells from nonproducer clone C-4 did not stimulate efficiently in MLTC after glutaraldehyde treatment.

Table VII presents further evidence for the inhibition of CTL generation by infectious Friend LV in MLTC: addition of 650–26 SFFU (obtained from a culture of clone B-2 cells) inhibited CTL generation in MLTC more than threefold. Addition of supernate from clone C-4 cells did not inhibit CTL generation in MLTC but actually seemed to potentiate it.

Discussion

The studies described in this report were centered around three aspects of the HFL/b tumor cell line: First, HFL/b cells were studied as a cell population which actively

TABLE VI

Comparison of x-irradiated and Glutaraldehyde-fixed HFL/b Cells as Stimulators in MLTC

Stimulating cells	Treatment	Stimulating cells per culture	CTL generation in MLTC (day 7)		
			Viable cells recovered	LU/10^6 cells	LU/culture
		$\times 10^{-6}$	$\times 10^{-6}$		
Clone B-2	5,000 rad	0.50	1.7	0	0
		0.17	1.6	5.9	9.4
		0.06	2.3	1.7	3.9
Clone B-2	Glutaraldehyde	0.50	2.9	3.3	9.6
		0.17	3.4	2.2	7.6
		0.06	2.8	1.2	3.3
Clone C-4	5,000 rad	0.50	1.8	1.4	2.5
		0.17	1.6	9.1	15.0
		0.06	2.0	4.0	8.0
Clone C-4	Glutaraldehyde	0.50	2.4	0.3	0.7
		0.17	2.6	0.8	2.1
		0.06	2.2	0.6	1.4

Clone B-2 cells (passage No. 122) and clone C-4 cells (passage No. 126) were either x-irradiated (5,000 rad) or fixed with 0.15% glutaraldehyde, and added in variable doses to 5 $\times 10^6$ HFL/b-immune BALB.B spleen cells in 2 ml culture medium. After 7 d incubation, each culture was assayed for the presence of CTL on ^{51}Cr-labeled clone B-2 cells in 6-h cytotoxicity assay at various lymphocyte:target cell ratios.

produced infectious Friend LV and eventually lost this capability. Second, cloned HFL/b cells were studied as targets for tumor-specific CTL. Third, HFL/b cells were studied as inducers of CTL differentiation in MLTC.

Arrest of virus production by cell lines previously producing infectious virus had been observed, but little information concerning this phenomenon was available. A fundamental question concerning such an observation is: did each of the cells in the population cease virus production more or less synchronously, or were cells present which either never had produced virus or ceased doing so as a rare event, and these nonproducers went on to become the exclusive survivors by virtue of an advantage in growth rate?

Our studies of the arrest of Friend virus production by the previously uncloned HFL/b tumor line provide a first-level answer to this question. Analysis of this line indicated that it contained both (*a*) cells giving rise to clones that maintained high levels of virus production far beyond the detection of arrest in the parent population and (*b*) cells giving rise to clones that produced very low levels of infectious virus and eventually lost all capacity to produce virus (Fig. 2). This finding suggests that, at the time of initial cloning, the population consisted of both high-producer and low-producer cells, though the former constituted the vast majority. Thus the arrest of virus production in this line was probably a result of a slight growth advantage of low-producer cells, allowing them to overgrow the producer cells; growth curve analysis, however, failed to reveal this small difference (data not shown). These observations also suggest that the occurrence of low-producer cells possessing growth

TABLE VII
Regulation of CTL Generation in MLTC by Tumor Cell Culture Supernates

Supernate added to MLTC*	CTL generation in MLTC (day 7)			Relative ratio‡
	Viable cells recovered	LU/10^6 cells	LU/culture	
	$\times 10^{-6}$			
MEM	1.7	8.5	14.4	1.00
B-2 (1/1)	1.8	1.7	3.1	0.22
B-2 (1/5)	2.0	2.8	5.6	0.39
B-2 (1/25)	1.8	2.5	4.4	0.31
B-2 (1/125)	1.7	7.0	11.9	0.83
C-4 (1/1)	1.5	25.0	37.5	2.60
C-4 (1/5)	2.0	12.5	25.0	1.74
C-4 (1/25)	2.0	9.3	18.6	1.29
C-4 (1/125)	1.4	7.7	11.1	0.77

MLTC were established with 5×10^6 HFL/b-immune BALB.B spleen cells, 0.2×10^6 x-irradiated (5,000 rad) clone C-4 cells, and 0.5 ml cell-free culture supernate. The vol was adjusted to 2.0 ml with MLTC medium, and the cultures were incubated for 7 d. CTL activity was detected by assaying each culture on ^{51}Cr-labeled clone B-2 cells at various lymphocyte:target cell ratios in a 6-h cytotoxicity assay.

* Cell-free culture supernates were prepared from 24-h cultures of 5×10^6 clone B-2 or clone C-4 cells, as described in Materials and Methods. Each supernate assayed in MLTC was also titrated in vivo for SFFV activity; clone B-2 supernate contained 1,100 SFFU/ml, and clone C-4 supernate was negative.

‡ Calculated as the quotient of each experimental value (in LU/culture) divided by 14.4 LU/culture.

advantage was a rare event among HFL/b cells. Superinfection of nonproducer cells by virus from producer cells would not be expected to occur, however, because low-producer and nonproducer clones were strongly viral glycoprotein gp69/71-positive (Table I), so that their virus receptor molecules would be blocked (21).

Arrest of virus production by HFL/b cells presumably represented a change of phenotype rather than of genotype. Consequently, nonproducer cells must retain the SFFV and FMuLV genomes. In fact, certain viral antigens continued to be expressed by nonproducer cells, and reactivation of virus expression has been achieved by treatment with iododeoxyuridine (10). In this context, Berkower et al. (22) have reported that producer B-2 and nonproducer C-4 clones showed differences in cytoplasmic viral RNA expression, and that arrest of virus production appeared to reflect deficient FMuLV transcription.

Serological tests for cell surface expression of various viral and *H-2* antigens in individual HFL/b clones failed to reveal any major differences between high- and low-producer clones. Both types of clones expressed high levels of viral glycoprotein gp69/71 at the cell surface, but low to undetectable levels of viral structural proteins p30 and p12. Consequently, the relatively high concentrations of p30 and p12 proteins previously detected in soluble extracts of producer HFL/b cells (10) were probably present in the form of cytoplasmic constitutents.

Our studies with HFL/b-specific CTL indicated that high- and low-producer HFL/b clones expressed at least one major antigenic determinant in common, which could be recognized by HFL/b-specific CTL. Two candidates for this antigen are

viral envelope glycoprotein gp69/71 and cellular FMR antigen, both governed by the helper FMuLV genome. Enjuanes et al. (23) have recently demonstrated that cell-surface viral glycoprotein gp69/71 antigen was a major determinant recognized by murine leukemia virus-specific CTL in another syngeneic tumor system. Viral structural *gag* proteins p30 and p12, on the other hand, appeared to be of minor importance for CTL recognition of target tumor cells in our HFL/b system, because all clones, regardless of virus-producer status, expressed low to undetectable amounts of these antigens.

It is probable that tumor-immune CTL comprise various independent subsets of cytotoxic cells that recognize antigenic determinants of different specificities particular to the tumor in question. Gillis et al. (4) have recently shown that a subpopulation of CTL directed to a SFFV-specific antigen can be obtained in a syngeneic tumor system involving immunity to Friend erythroleukemia cells. Another aspect of SFFV-specific antigens has been described by Risser (24) with serological studies that indicated that SFFV-specific antigens are similar to tissue-specific differentiation antigens. The latter appear to be expressed selectively in a subpopulation of hemopoietic cells in strains of mice carrying the $Fv\text{-}2^s$ allele. HFL/b-immune CTL probably include cytotoxic cells specific for antigens determined by both the helper FMuLV and the SFFV genomes.

It was unexpected that virus-producer HFL/b cells were markedly more sensitive to lysis by syngeneic CTL than were nonproducer cells. Because both types of cells were comparably susceptible to anti-$H\text{-}2^b$ CTL, no clone was inherently resistant to CTL-mediated lysis. Although studies of Meruelo (25) suggested that increased expression of *H-2* antigens might result in increased susceptibility to syngeneic tumor-immune CTL, we detected no difference between high and low producer clones with respect to H-2.2 antigen expression. Finally, cold target cell competition assays showed that both high producer and nonproducer HFL/b cells expressed tumor antigen(s) in amounts sufficient to compete comparably for CTL-mediated lysis. Thus, the heightened expression of target antigens in high-producer HFL/b cells did not render them better competitor cells. The identity of these target antigens remains to be better defined. Our results suggest that they are not all identical to the FMR and viral glycoprotein gp69/71 antigens detected serologically, because our producer and nonproducer clones expressed roughly equivalent amounts of the latter. The use of monoclonal antibodies might help define the relevant target antigens in the future.

Having established that active Friend LV production by clone HFL/b cells correlated with a high susceptibility to attack by HFL/b-immune CTL, we proceeded to investigate the ability of the same HFL/b clones to induce the generation of HFL/b-specific CTL in secondary MLTC. Our results indicated that low-producer clones were capable of stimulating CTL differentiation over a relatively wide range of responding spleen cell-to-stimulating tumor cell ratios. High-producer clones were also efficient stimulators of CTL differentiation when added in small numbers to the lymphocyte cultures; however, the same cells inhibited CTL differentiation at higher doses. Further experiments revealed that this inhibitory capacity of high producer cells required metabolically active stimulating cells, and that it could be partially reproduced with nonproducer cells by adding 50 or more SFFU of Friend LV to the lymphocyte cultures. This circumstantial evidence suggested that in spite of x-irradiation, high producer cells continued to produce virus for a period of time in MLTC, and that the virus released interfered with CTL differentiation in vitro. An analogous

pattern of interference by Friend LV on the differentiation of immunoglobulin-producing B cells in vitro was observed by Kamo et al. (26), who found evidence for the active inhibition of specific immunoglobulin production by free Friend LV secreted in the lymphocyte cultures by infected spleen cells.

The totality of these experiments thus indicates that Friend LV-induced tumor cells can vary radically in their ability to produce virus, that those tumor cells actively producing large amounts of virus are the most susceptible to attack by tumor-specific CTL, and that active Friend LV production can inhibit CTL differentiation in MLTC. In general, these facts imply that LV-induced tumors can affect the immune response directed against them by modulating their capacity to produce infectious LV and, concomitantly, modulating their antigenic identity.

Summary

The HFL/b tumor cell line, induced by Friend erythroleukemia virus in BALB.B mice, was used to study the relation between virus production or nonproduction and the antigens recognized by Friend virus-specific cytolytic T lymphocytes (CTL). Analysis of clones and subclones of these tumor cells revealed a high degree of heterogeneity with respect to the production and release into culture fluids of infectious Friend virus in vitro, ranging from high levels to low or undetectable levels of virus production.

Although no major differences could be detected among the antibody-defined serotypes of the various clones, the susceptibility of cells of individual HFL/b clones to attack by Friend virus-specific CTL varied widely, and those clones which produced large amounts of infectious virus provided the most sensitive target cells. It was also apparent that production of infectious Friend virus was inhibitory to CTL generation in syngeneic mixed leukocyte-tumor cell cultures. Friend erythroleukemia virus-producing cells thus appeared to interact in a complex manner with the host CTL response by modulating their production of infectious Friend virus.

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