PROPERTIES OF CELL LINES DERIVED FROM TUMORS INDUCED BY FRIEND VIRUS IN BALB/c AND BALB/c-*H-2*^{b} MICE*

By HERBERT A. FREEDMAN‡ and FRANK LILLY

(*From the Department of Genetics, Albert Einstein College of Medicine, Bronx, New York 10461*)

Genes located at the *Fv-1*, *Fv-2*, and *H-2* chromosomal regions are the major polymorphic systems in the mouse which govern the pathological consequences of inoculation with Friend virus (FV) (reference 1 and footnote 1). *Fv-1* and *Fv-2* appear to govern susceptibility to the helper virus component and to the defective SFFV[1] component, respectively, of the FV complex, both of which are necessary for induction of the characteristic erythroleukemic disease. The *H-2*-associated gene, on the other hand, appears to exert its influence relatively late during the development of the disease syndrome, since its effects are demonstrable only after cellular infection and virus proliferation have occurred.

Because early experiments showed that the *H-2*^{d} haplotype of BALB/c mice favored the occurrence of the FV disease whereas the *H-2*^{b} haplotype of C57BL mice was associated with relative resistance to the disease (2), the congenic BALB/c-*H-2*^{b} (BALB.B) strain was bred in our laboratories. Comparisons of BALB/c and BALB.B mice showed that they are equally susceptible to FV by the criterion of spleen focus induction, which is a measure of cellular infectivity, but by the criterion of splenomegaly induction BALB.B mice require about 10–20 times as much FV to produce the same pathological response as that of BALB/c mice (3).

In order to advance our studies of the mechanism of the *H-2* effect on the FV disease, we have embarked on a series of experiments designed to examine the properties of tumor cells induced with FV in BALB/c and in congenic BALB.B mice subsequently grown in culture. We now report some similarities and differences between two such cell lines. Although the differences we have observed cannot yet be attributed with certainty to the differences in *H-2* genotype, they have provided us with a new frame of reference for further studies of this problem.

* Supported by contract NO1 CP NCI 33249 from the National Cancer Institute.

‡ Fellow of the Leukemia Society of America, Inc.

[1] *Abbreviations used in this paper:* BALB.B, BALB/c-*H-2*^{b} congenic partner to BALB/c (*H-2*^{d}); FV, Friend virus complex; HFL/b-sc, solid subcutaneous tumor derived from FV-infected BALB.B spleen fragments; HFL/b-tc, HFL/b-sc grown in tissue culture; HFL/d-as, HFL/d-tc grown in ascitic form in vivo; HFL/d-sc, solid subcutaneous tumor derived from FV-infected BALB/c spleen fragments; HFL/d-tc, HFL/d-sc grown in tissue culture; LLV-F, lymphatic leukemia isolated from FV; MEM-Earle's, MEM (Eagle's) with Earle's salts; MolLV, Moloney leukemia virus; PBS, phosphate-buffered saline; PFU, plaque-forming units; SFFU, spleen focus-forming units; SFFV, defective component of FV which in the presence of helper viruses induces spleen foci in susceptible hosts.

Materials and Methods

Mice. With the exception of some of the animals used for virus titrations, all mice were from our own colony of highly inbred mouse strains. The congenic mouse strain referred to as BALB.B, was established from the (BALB/c × C57BL/10)F_1 cross. This strain was inbred after 13 consecutive backcrosses to the BALB/c parental strain with selection in each generation for the *H-2*^{b} haplotype of the C57BL/10 ancestor. Thus, any differences observed between BALB/c and BALB.B mice can be attributed with high probability to a gene or genes in the immediate vicinity of the *H-2* region of linkage group IX (chromosome 17).

For virus titrations, DBA/2Ha, random-bred Ha/ICR Swiss and BALB/c mice were obtained from the West Seneca Laboratories, Buffalo, N. Y. Animals were usually 6- to 10-wk old at the time of virus inoculation.

Viruses. The variant N→NB-tropic strain of FV was used throughout these experiments. This virus strain was derived from the original N-tropic FV by forced passage in BALB/c mice (4). N→NB-tropic FV is equally infective in DBA/2 (*Fv-1*^{n}) and BALB/c (*Fv-1*^{b}) mice and their F_1 hybrids. Preparations of FV were obtained from the greatly enlarged spleens of BALB/c mice infected 2 wk previously with N→NB-tropic FV from a syngeneic donor. The spleens were homogenized in nine times their weight of cold phosphate-buffered saline (PBS). After slow centrifugation at 4°C to remove large particulate matter from the homogenate, the supernate was recentrifuged for 4 min at 7,000 *g*. The resulting supernate was stored in small ampules at −70°C and served as the basic virus preparation. When assayed in BALB/c mice, such preparations usually show a titer of about 5×10^4 spleen focus-forming units (SFFU)/ml.

A lymphatic leukemia virus was isolated from N-tropic FV (LLV-F) by passage into newborn C57BL (*Fv-1*^{b}) mice followed by blind passage in weanling Ha/ICR swiss mice (5). Conversion to NB tropism occurred during five subsequent serial passages in BALB/c mice. All LLV-F stocks were free of detectable spleen focus-forming activity. Moloney leukemia virus (MolLV) was obtained in 1964 from Dr. J. B. Moloney, National Cancer Institute, Bethesda, Md. This virus was considered NB tropic after four passages in BALB/c mice. Stocks of LLV-F and MolLV were prepared as 20% perfusates of leukemic BALB/c spleens. Enlarged spleens were pooled and perfused with McCoy's medium containing 3% fetal calf serum, and the fluids were clarified at 2,400 *g* for 10 min and stored at −196°C. Stocks of LLV-F and of MolLV were titrated for their helper activity for SFFV by the method of Steeves et al. (6) and showed 8×10^4 and 9×10^3 helper U/ml, respectively.

Virus Assays. FV was routinely titrated in vivo by the spleen focus assay (7). Diluted samples were injected into the lateral tail veins of mice in 0.5-ml vol. 9 days later the spleens of the recipients were excised, fixed in Bouin's solution, and examined for discrete, macroscopic, focal lesions on the splenic surfaces. Virus titers were expressed in SFFU per milliliter.

Titrations in vitro of virus samples were performed by the XC plaque assay (8). For this assay, embryo fibroblasts of the NIH Swiss (*Fv-1*^{n}) and BALB/c (*Fv-1*^{b}) strains were obtained from Microbiological Associates, Inc., Bethesda, Md. In addition embryo fibroblasts of the BALB/c and DBA/2 (*Fv-1*^{n}) strains were obtained from animals in our own colony. Virus titers were expressed in plaque-forming units (PFU) per milliliter.

Tissue Culture. All cell lines grown in culture were established from their respective subcutaneous solid tumors. These lines consisted of nonadherent cells maintained in stationary suspension culture. Adherent cells were discarded. Essentially, tissue fragments from the various tumors were minced in Eagle's minimum essential medium (MEM) with Earle's base plus glutamine (MEM-Earle's), filtered through sterile gauze, and the resulting cell suspension washed five to six times in MEM-Earle's. These cells were resuspended at a concentration of 5×10^6 cells/ml in complete growth medium: MEM-Earle's containing 20% fetal calf serum, extra L-glutamine (0.58 mg/ml total), 100 U/ml penicillin, and 100 mcg/ml streptomycin. T-30 flasks were seeded with 5 ml of the cell suspensions and incubated at 37°C. After 24 h each culture was diluted fivefold with complete growth medium and the cells redistributed in 5-ml vol into new T-30 flasks. The cells were then maintained at a concentration of 5×10^5 cells/ml by periodic dilution with fresh growth medium. Within 3 wk the cells in successful cultures appeared to be well adapted to growth in vitro. Thereafter, the cells were maintained in complete growth medium containing 10% fetal calf serum and 0.292 mg/ml L-glutamine by planting 5-ml vol containing 5×10^4 cells/ml in T-30 flasks followed by subculturing when the cell concentration reached 5×10^6 cells/ml (approximately 5 days). This same growth medium was used for all cocultivation experiments as well.

Results

Derivation of BALB/c Tumor: HFL/d. 25–28 days after infection of a BALB/c female mouse with N→NB-tropic FV, washed fragments of the greatly enlarged spleen were transplanted subcutaneously to syngeneic hosts. Such implants usually enlarged slowly but progressively. In the early transplant generations, the recipients showed a concomitant primary FV-disease syndrome as well as growth of the transferred tumor cells. For this reason it was important to transfer the tumor at 7- to 14-day intervals, even though the tumors were relatively small at this time. In later transplant generations, the transfer of infectious virus with the tumor cells appeared much diminished, since the accompanying primary disease syndrome seemed weaker or occasionally nonexistent. In addition, the growth of the transplanted tumors appeared to become more rapid in later generations. Grossly, this tumor resembled those produced originally by Buffett and Furth (9) and by Friend and Haddad (10) by similar methods. This tumor has been designated HFL/d-sc; "d" refers to the *H-2*^{d} type of the mice from which this tumor was derived, and "sc" refers to subcutaneous passage.

Derivation of BALB.B Tumor: HFL/b. The transplantable subcutaneous tumor, HFL/b-sc, was induced in a congenic BALB.B female mouse. This tumor was established in essentially the same manner as the *H-2*-congenic HFL/d-sc tumor. However, it is notable that transfers of BALB.B-infected spleen fragments showed only transient growth followed by tumor rejection in normal syngeneic hosts, so that the tumor line could not be established in these hosts. When the recipients were rendered anemic by bleeding (~0.3 ml three times a week), beginning the day of transplantation, the transplants showed much more reliable progressive growth (Table I). Grossly, progressively growing HFL/d-sc and HFL/b-sc tumors were indistinguishable.

Characteristics of FV-Induced Tumor Cells In Vitro. Tissue cultures were initiated from HFL/d-sc tumors on two separate occasions, separated by a 5-mo interval, giving rise to cell lines, HFL/d-tc and HFL/d-tc-II. These cell lines have shown indistinguishable properties by all criteria applied to their study, and they will not be discussed separately. The HFL/d-tc cells have been maintained in continuous culture for 2.5 yr at present, and their properties have been stable

TABLE I

Effect of Bleeding on the Growth of HFL/b-sc Solid Implants in BALB.B Mice

Passage no.	Normal		Bled	
	Tumor growth	Tumor regression	Tumor growth	Tumor regression
6	5/5*	5/5‡	4/4*	0/4‡
8	6/6	4/6	4/4	0/4
13	10/10	4/10	4/4	0/4

* Number of mice with palpable tumors (minimum diameters, 5 mm)/number of mice inoculated.

‡ Number of mice with no palpable tumors/number of mice originally showing palpable tumors (minimum diameter, 5 mm).

since about the 3rd mo in culture. The cells grow in suspension with or without agitation, and neither extensive clumping nor flattened fibroblastic or epithelioid elements have been observed. Actively growing cells are spherical, range in size from 10–20 μm, and have a doubling time of approximately 14 h.

Subcutaneous injection of as few as 1×10^3 HFL/d-tc cells produced tumors in syngeneic hosts within a maximum of 3 wk (Table II). Grossly, these tumors were indistinguishable from the original HFL/d-sc tumor. Intraperitoneal inoculation of 10^4–10^6 cells resulted in cellular proliferation in the ascitic form in 20–60% of the inoculated syngeneic hosts, which then died within 2–3 wk.

Tissue cultures initiated from the HFL/b-sc tumor have been maintained in serial passage for approximately 2 yr. The resultant cell line, HFL/b-tc appeared identical to the HFL/d-tc cell lines. However, implants of as many as 10^7

TABLE II

Growth of Palpable Tumors in BALB/c Mice Receiving HFL/d-tc Cells Subcutaneously

Passage no.	No. of cells inoculated						
	10^7	10^6	10^5	10^4	10^3	10^2	10^1
9		4/4*					
13		9/9					
15	5/5						
18		8/8					
21		7/7					
56		6/6	4/4	3/5	2/5	0/5	0/5

* Number of mice with palpable tumors (minimum diameter, 5 mm)/number of mice inoculated.

HFL/b-tc cells rarely induced progressively growing tumors in normal syngeneic hosts. Rather, it was necessary to render the mice anemic by bleeding in order to achieve tumor growth in vivo, as noted previously with regard to the HFL/b-sc tumor line. A detailed study of the growth properties in vivo of HFL/d-tc and HFL/b-tc cells will be the subject of a subsequent paper.

Wright-stained preparations (11) of HFL/d-tc and HFL/b-tc cells revealed at least three cell types. The most abundant cell appeared to be a proerythroblast exhibiting a high nucleocytoplasmic ratio, deep blue-staining cytoplasm, nuclear hyperchromatism, and coarse chromatin aggregates. Although most cells contained a single round nucleus, occasional binucleate forms were present.

The second cell type appeared somewhat larger than the proerythroblast with paler staining nucleus and cytoplasm. Nuclear chromatin was arranged in a fine, open meshwork containing two to four large irregularly shaped nucleoli. Such cells resemble very closely those described by Friend et al. (11) and referred to as primitive reticulum or "stem" cells. Mitoses were frequently observed among both proerythroblasts and reticulum cells.

Erythroblasts in more advanced stages of maturity were present as well, although reticulocytes or fully mature anucleate erythrocytes were absent. Accordingly, the most advanced stage observed appeared identical to the basophilic erythroblasts described by Friend et al. (11) and Ikawa et al. (12). This

third cell type appeared smaller than the proerythroblast and displayed a strongly basophilic cytoplasm. Nuclei appeared condensed with coarse chromatin clumping and marked decrease in nucleocytoplasmic ratio. The proportion of stem cells to proerythroblasts and erythroblasts appeared to vary appreciably with the density of the cell population, as well as from passage to passage.

Friend Virus Production by HFL/d Cells. Supernates of 10% saline homogenates of HFL/d-sc tumors showed marked activity in the spleen focus assay. The extracts yielded about 10^3 SFFU/ml in both *Fv-1*^{n} (DBA/2 and Ha/ICR) or *Fv-1*^{b} (BALB/c) hosts, indicating the presence of NB-tropic FV (Table III, A1). This

TABLE III

Occurrence of Friend Virus in Tumors, Culture Fluids, and Hosts of HFL/d Cells

Tissues tested		*Fv-1* type of test cells or mice	Virus titer	
Source	Preparation		XC cell assay	Spleen focus assay
			PFU/ml	*SFFU/ml*
A				
1. HFL/d-sc tumor	Ext*	n	13.6×10^4	0.9×10^3
		b	3.8×10^4	1.1×10^3
2. HFL/d-tc cell culture	Sup‡	n	0	0
		b	0	0
B				
1. HFL/d-tc tumor (grown s.c. in host infected with NB-tropic MolLV)	Ext	b		8×10^3
2. HFL/d-tc cells (cocultivated with NB-tropic LLV-F-infected embryo cells)	Sup	b		2×10^2
3. HFL/d-tc cells (cocultivated with NB-tropic MolLV-infected embryo cells)	Sup	b		1×10^2
C				
1. Spleen of BALB/c host of HFL/d-as1 cells (passage no. 4 in vivo)	Ext	n	1.6×10^6	7×10^5
		b	6.9×10^6	4×10^5
2. Spleen of BALB/c host of HFL/d-as2 cells (passage no. 3 in vivo)	Ext	n	2.0×10^6	1.1×10^6
		b	2.9×10^6	1.3×10^6
3. HFL/d-as2 cells returned to culture (passage no. 2 in vitro)	Sup	b		0
4. HFL/d-as2 cells returned to culture (passage no. 13 in vitro)	Sup	n	0	
		b	0	

* Supernate of 10% tissue homogenate in PBS.
‡ Culture fluid supernate.

finding is consistent with the fact that the tumor was originally induced by N→NB-tropic FV.

When these tumor cells were grown in culture (HFL/d-tc), FV detectable by the spleen focus assay in vivo was revealed in supernatant fluids. Virus persisted for at least 13 weekly passages, showing titers in the range of 10^2–10^3 SFFU/ml culture supernate 6 days after subculture. At the 16th and subsequent passages FV could no longer be detected in supernates by the spleen focus assay. These supernates were also virus negative when assayed in vitro by the XC method

(Table III, A2) and showed no helper activity in vivo for the helper-dependent SFFV (6).

The cell line, HFL/d-tc-II, derived from the HFL/d-sc tumor at a later date, was indistinguishable from HFL/d-tc. When assayed in homologous hosts by the spleen focus method, primary cultures of this cell line showed 1×10^3 SFFU/ml supernatant fluid, and secondary cultures, 7×10^2 SFFU/ml. By the third passage, however, virus could no longer be detected by the spleen focus assay. No XC-active virus was detected in subsequent culture supernates assayed on either *Fv-1*^{n} or *Fv-1*^{b} embryos fibroblasts.

FV Production by HFL/b Cells. Supernatant fluids harvested from HFL/b-tc cultures at the sixth passage generation showed NB-tropic FV at a concentration of 4.3×10^2 SFFU/ml when assayed in vivo. When assayed in vitro by the XC method this same preparation showed NB-tropic virus at a concentration of 2.0×10^3 PFU/ml (Table IV). In subsequent passage generations, culture fluids which have been tested for virus either in vitro or in vivo have invariably shown the presence of virus at approximately these same levels. In contrast with "nonproducer" HFL/d-tc cells, HFL/b-tc cells produce complete FV, i.e., defective SFFV plus helper virus.

Recovery of Friend Virus From HFL/d Cells. Although HFL/d-tc cells do not produce infectious FV in amounts detectable in either the spleen focus or the XC assays, the cells might nevertheless still possess the genomes of either defective SFFV or helper virus or both in unexpressed form. In an attempt to recover the SFFV component of FV in infectious form, HFL/d-tc cells harvested from the 22nd passage were inoculated subcutaneously in BALB/c hosts which have been infected 5.5 mo earlier with NB-tropic MolLV. By day 15 all recipients showed palpable tumors, 0.5–1.0 cm in diameter. An extract of these tumors (supernate of 10% PBS homogenate) assayed in vivo by the spleen focus method showed 8×10^3 SFFU/ml (Table III, B1). Since MolLV itself shows no activity in the spleen focus assay, it appears that SFFV was rescued from HFL/d-tc cells during their residence in helper virus-infected hosts.

Attempts to recover FV in vitro were made as well. Syngeneic embryo fibroblasts infected in culture with NB-tropic MolLV were grown as monolayers and allowed to reach approximately 80% confluence. At this point HFL/d-tc cells taken from the 34th passage were added to these fibroblast cultures. After cocultivation for 7 days, supernates were removed and tested for spleen focus-forming activity. These tests showed NB-tropic virus at a concentration of approximately 24 SFFU/ml, confirming in vitro the rescue of SFFV by helper virus (MolLV) already demonstrated in vivo.

Observations during the course of cocultivation suggested that a number of HFL/d-tc cells adhered to areas of the fibroblast monolayers. These adherent cells were not dislodged by vigorous agitation. With this in mind, cocultivation was repeated using either NB-tropic MolLV or NB-tropic LLV-F-infected syngeneic fibroblasts. All fibroblasts were infected in vitro. This time, however, during the course of cocultivation with HFL/d-tc cells harvested from the 40th passage generation, supernatant fluids were decanted daily, and replaced with fresh growth media. Although this procedure resulted in the loss of many nonadherent cells, supernatant fluids tested 7 days after the initiation of

TABLE IV

Occurrence of Friend Virus in Culture Supernates from Serially Transferred HFL/b-tc Cells

Passage no.	*Fv-1* type of test cells or mice	XC cell assay	Spleen focus assay
		PFU/ml × 10^2	*SFFU/ml* × 10^2
4	b		>2.0
5	b		>2.0
6	n	24	5.8
	b	15	2.7
14	b		>2.0
20	b		>2.0
22	n	2.8	
	b	3.2	
25	b		4.8
28	b		9.6
38	b		>2.0
61	b		>2.0
95	b		>2.0
97	b		3.0
98	b		>2.0
102	b		>2.0
111	n, b*	>2.0	
118	n		1.5
	b		4.0
121	b	28	

* III 6A cells (3T3 cell line derived from a wild mouse by Dr. W. P. Rowe and donated to us by Dr. T. Pincus, Sloan-Kettering Institute for Cancer Research, New York; these cells do not show an apparent *Fv-1* type, but rather they are equally efficient hosts for both N- and B-tropic viruses).

cocultivation with MolLV-infected fibroblasts showed 100 SFFU/ml. Supernatant fluids harvested from parallel cultures of HFL/d-tc cells with LLV-F-infected fibroblasts contained 200 SFFU/ml (Table III, B2 and B3). Supernates from parallel control cultures of HFL/d-tc cells with normal, uninfected fibroblasts contained no spleen focus-forming activity.

Another method of recovering infectious FV from HFL/d-tc cells was encountered during the course of these experiments. As noted previously, inoculation of these cells intraperitoneally into syngeneic hosts results in the growth of the cells as an ascitic tumor in a portion of the recipients. A line of tumor serially passaged in this ascites form, HFL/d-as1, was initiated with an intraperitoneal inoculum of 2.5×10^5 HFL/d-tc cells from the 21st passage in culture. Subsequent passages in vivo of the HFL/d-as1 line were performed with whole ascites fluid which contained cells morphologically indistinguishable from the original HFL/d-tc cell line. Hosts harboring the fourth passage generation of these cells (71 days after initiation of the ascites line) unexpectedly showed marked splenomegaly. When a 10% extract in PBS of one of these enlarged spleens was assayed in vivo for FV by the spleen focus assay, a titer of 5.5×10^5 SFFU/ml was demonstrated. Assay of the same preparations in vitro by the XC method revealed 4.3×10^6 PFU/ml. This virus was NB-tropic (Table III, C1).

In order to confirm this phenomenon, 1×10^5 HFL/d-tc cells of the 33rd passage were transplanted intraperitoneally to syngeneic hosts. Serial passage in vivo resulted in the establishment of the ascitic HFL/d-as2 cell line. This time hosts harboring the third serial passage generation (57 days after initiating of the cell line) displayed splenomegaly. Extracts of these spleens showed NB-tropic FV at a concentration of 1.2×10^6 SFFU/ml when assayed in vivo and NB-tropic virus at a concentration of 2.5×10^6 PFU/ml when assayed by the XC method (Table III, C2).

It should be emphasized that, although mice carrying HFL/d-as cells demonstrate FV in their spleens, the HFL/d-as cells recovered from the peritoneal cavity did not appear to be actively producing virus. When HFL/d-as cells, harvested from the peritoneal cavity of viremic hosts, were carefully washed before inoculation into the peritoneal cavity of normal syngeneic hosts, no splenomegaly was observed until the third serial intraperitoneal passage. Similarly, HFL/d-as2 cells maintained in vivo for six passage generations and then returned to culture showed no virus activity when supernatant fluids were assayed in vivo or in vitro (Table III, C3 and C4). When after a total of six serial passages in vitro, these cells were returned to the peritoneal cavity of syngeneic hosts and passed serially, host splenomegaly did not reappear until approximately 87 days after reinitiation of intraperitoneal passage (fifth passage generation).

Discussion

We have established cell lines in culture from tumors derived from the enlarged spleens of FV-infected BALB/c ($H\text{-}2^d$) and congenic BALB.B mice. Morphologically these cell lines, as well as the tumors from which they were derived, resemble those produced with similar techniques in other laboratories (9–16). Cultures of these cells are composed of red cell precursors in various stages of differentiation. This description is consistent with the observation that treatment of these cultures with dimethylsulfoxide results in an increase in both the number of benzidine-positive cells and the total amount of detectable hemoglobin (reference 17, and M. Adesnick, unpublished observation). Thus, the hematopoietic potential of these cultures has been established. The malignancy

potential of these cells was demonstrated by the formation and progressive growth in situ of solid tumors after inoculation into syngeneic hosts.

When BALB/c tumors (HFL/d-sc) and their cultured counterparts (HFL/d-tc) were assayed for production of FV, only solid tumor extracts and supernates from early passages in culture gave positive results. Subsequent passages in vitro revealed no infectious virus by either XC or spleen focus assays. In contrast, supernates of cultures derived from BALB.B tumors persistently exhibited infectious virus detectable in both XC and spleen focus assays. The nonproducer status of HFL/d-tc cells corroborates and expands upon the results of Fieldsteel et al. (15, 18, 19), who have also found that tumor cells derived from FV-infected BALB/c mice fail to produce virus in vitro.

In addition to infectivity assays, demonstration of virus-like particles with a density of 1.16 g/cm^3 and virus-associated reverse transcriptase activity confirmed the presence of virus in HFL/b-tc culture supernates of the 28th, 38th, and subsequent passage generations. Neither virus-like particles nor reverse transcriptase activity were found in HFL/d-tc cultures (M. Adesnick, unpublished observations). No intra- or extracellular virus-like particles could be seen electron microscopically in thin sections of HFL/d-tc cells. In contrast similar preparations of HFL/b-tc cells showed C-type particles, both extracellularly and budding from the cell surface (S. Panem, unpublished observation).

The cell lines that we have established differ genotypically from each other only with respect to the *H-2* region of chromosome 17 (linkage group IX). Nonproducer HFL/d-tc cells originated from a BALB/c mouse and possess the $H\text{-}2^d$ haplotype; producer HFL/b-tc cells are of congenic BALB.B origin and possess the $H\text{-}2^b$ haplotype derived from C57BL/10 mice. It is tempting to speculate that this genotypic difference is the basis for the marked difference in virus production noted between the two cell lines, but at this time it remains possible that other factors are involved in these observations. Further studies will be required to elucidate this point.

FV is a complex consisting of a spleen focus-forming component (SFFV) and an associated helper (5, 19, 20). It is the SFFV which confers upon the virus complex the ability to induce spleen foci and erythroleukemia. The presence of the SFFV genome within nonproducer HFL/d-tc cells was demonstrated by successful recovery of complete FV when these cells were grown in the presence of either LLV-F- or MolLV-infected cells which can act as helpers. Recovery of FV was successful both in vivo and in culture. This was accomplished in vivo by subcutaneous transplantation of HFL/d-tc cells to syngeneic animals previously infected with MolLV. Recovery in vitro was afforded by cocultivation of HFL/d-tc cells with syngeneic embryo fibroblasts infected in vitro with LLV-F or MolLV. Using analogous techniques both in vivo and in vitro, Fieldsteel et al. (21) have also shown that their FV-transformed, nonproducer BALB/c cell line contains the SFFV genome.

SFFV appears to be defective in that it cannot actively proliferate in the absence of its associated helper virus. Thus it was conceivable that the nonproducer status of HFL/d-tc cells was due to the loss of the helper virus genome from these cells. However, this does not appear to be the case. The presence of the helper virus genome within HFL/d-tc cells was suggested by the

recovery of complete FV from the spleens of syngeneic hosts in which HFL/d cells (HFL/d-as) were passaged intraperitoneally. High titers of FV were demonstrable in the spleens of hosts harboring HFL/d-as cells at the third and subsequent serial intraperitoneal passages. In this context it is critical to note that the FV recovered was NB tropic, as was the original virus used to induce the HFL/d cell line. Since no exogenous helper virus was added, and since endogenous NB-tropic viruses are not known to occur in nature, it appears that the helper virus genome was indeed contained within nonproducer HFL/d-tc cells.

It should be emphasized, however, that although mice harboring HFL/d-as cells demonstrate FV in their spleens, the HFL/d-as cells themselves did not appear to be actively producing FV. This was evidenced by the fact that HFL/d-as cells did not produce FV in culture, nor did washed HFL/d-as cells induce splenomegaly upon intraperitoneal inoculation into normal syngeneic hosts until several serial passages in vivo had been carried out.

These observations indicate that most HFL/d cells were not converted to active FV producers by serial passage in the ascitic form. Rather, it appears that an extremely small percentage of HFL/d cells have been converted to FV producers or are continuously producing FV in quantities too small to detect by infectivity assays. This small amount of virus could then be amplified by replication in susceptible host cells. Another possibility is that a small number of nonproducer HFL/d cells passaged intraperitoneally find their way to the host spleen where their viral genomes are passed to new red cell precursors, establishing a primary infection.

In all cases in which FV was recovered from nonproducer HFL/d-tc cells, either host cells or mouse embryo fibroblasts were present. Thus, the possibility exists that HFL/d-tc cells cannot synthesize FV by themselves but can do so only in cooperation with a "helper cell." Lala has found that Ehrlich ascites cells occasionally fuse with host cells in the peritoneal cavity (22). It is possible that intraperitoneally passaged HFL/d-tc cells fuse with host cells in situ. Fusion of HFL/d cells with host cells could perhaps result in expression of the viral genomes in the form of infectious particles.

In these studies we have employed both the spleen focus and XC assays. The spleen focus assay was used because it is specific for complete FV, i.e., spleen foci are induced by SFFV only in the presence of helper virus. The XC assay, on the other hand, is capable of measuring helper virus even in the absence of SFFV. Since the results obtained by both methods were comparable, both components of FV were expressed coordinately.

Curiously, we were unable to establish the BALB.B tumor (HFL/b-sc) in normal BALB.B hosts. Fragments of FV-infected BALB.B spleens failed to grow progressively upon transplantation to normal BALB.B mice. However, when BALB.B recipients were rendered anemic by bleeding during the course of tumor derivation, the BALB.B tumor was readily established. Transplants of BALB.B tumors (HFL/b-sc) or their cultured counterparts (HFL/b-tc) consistently regressed except in anemic hosts.

Although the mechanism of this effect of bleeding on tumor growth in BALB.B hosts is as yet undetermined, two possible explanations can be postulated. One explanation might be that the anemia induced by bleeding results in increased

levels of circulating erythropoietin which in turn stimulates the growth of transplanted FV-transformed red cell precursors. This possibility would agree with earlier studies indicating that erythropoietin increases susceptibility to FV by increasing the number or the susceptibility of target cells (i.e., erythropoietin-sensitive primitive erythroid cells) before transformation (23). Another possible explanation is that bleeding not only decreases humoral antibody levels but also removes migrating immunocytes which reject the tumor cells. In fact, both mechanism may be operating in concert.

Summary

Cell lines have been established in culture from Friend virus-induced tumors of BALB/c (*H-2*^{d}) and congenic BALB/c-*H-2*^{b} (BALB.B) origin. Spleens from virus-infected hosts in the terminal stages of erythroleukemic disease provided tissues for the establishment of subcutaneously transplantable tumors of both strains. Subsequently cells of these tumors were introduced into culture and passed serially. Complete, infectious Friend virus (FV) has been routinely recovered from culture supernates of BALB.B tumor cells (HFL/b) throughout its 2-yr passage history. However, after only a few transfer generations in culture BALB/c tumor cells (HFL/d) became nonproducers of virus detectable in either the spleen focus assay in vivo or the XC assay in vitro. Nonproducer HFL/d cells possessed the complete genomes of the components of the FV complex, since FV could be recovered from them either by cocultivation with helper virus-infected syngeneic embryo fibroblasts or by serial passage in the ascitic form in normal, syngeneic adult hosts.

We would like to take this opportunity to thank Mr. Leo Spaccavento for his technical assistance in performing XC assays, Mrs. Harriel Martinez for her assistance in the breeding and maintenance of our colony of highly inbred mouse strains, and Mrs. Ida Shapiro for her kind assistance in the completion of this manuscript. Dr. R. A. Steeves has provided helpful comments on the work and the manuscript and has supplied us with our NB-tropic helper virus preparations (LLV-F and MolLV).

Received for publication 19 March 1975.

References

1. Lilly, F., and T. Pincus. 1973. Genetic control of murine viral leukemogenesis. *Adv. Cancer Res.* **17**:231.
2. Lilly, F. 1968. The effect of histocompatibility-2 on response to the Friend leukemia virus in mice. *J. Exp. Med.* **127**:465.
3. Lilly, F. 1972. Antigen expression on spleen cells of Friend virus-infected mice. *In* RNA Viruses and Host Genome in Oncogenesis. P. Emmelot and P. Bentvelzen, editors. North-Holland Publishing Co., Amsterdam, The Netherlands. 229.
4. Lilly, F. 1967. Susceptibility to two strains of Friend leukemia virus in mice. *Science (Wash. D. C.).* **155**:461.
5. Steeves, R. A., R. J. Eckner, M. Bennet, and E. A. Mirand. 1971. Isolation and characterization of a lymphatic leukemia virus in the Friend virus complex. *J. Natl. Cancer Inst.* **46**:1209.
6. Steeves, R. A., R. J. Eckner, E. A. Mirand, and R. L. Priore. 1971. Rapid assay of murine leukemia virus helper activity for Friend spleen focus-forming virus. *J. Natl. Cancer Inst.* **46**:1219.
7. Axelrad, A. A., and R. A. Steeves. 1964. Assay for Friend leukemia virus: rapid

quantitative method based on enumeration of macroscopic spleen foci in mice. *Virology*. **24**:513.

8. Rowe, W. P., W. E. Pugh, and J. W. Hartley. 1970. Plaque assay techniques for murine leukemia viruses. *Virology*. **42**:1136.
9. Buffett, R. F., and J. Furth. 1959. A transplantable reticulum cell sarcoma variant of Friend's viral leukemia. *Cancer Res.* **19**:1063.
10. Friend, C., and J. Haddad. 1959. Local tumor formation with transplants of spleen or liver of mice with a virus-induced leukemia. *Proc. Am. Assoc. Cancer Res.* **3**:1.
11. Friend, C., G. B. Rossi, M. C. Patuleia, and E. DeHarven. 1967. The cloning of tissue culture lines of murine virus-induced (Friend) leukemic cells. *In* Subviral Carcinogenesis Monograph of the First International Symposium on Tumor Viruses. Y. Ito, editor. NISSHA, Kyōto, Japan. 408.
12. Ikawa, Y., and H. Sugano. 1963. An ascites tumor derived from early splenic lesion of Friend's disease: A preliminary report. *GANN Jap. J. Cancer Res.* **57**:641.
13. Odaka, T., and Y., Ikawa. 1968. Friend virus-induced transplantable tumors of C57BL/6 origin containing unusually minute amounts of infectious virus. *Int. J. Cancer.* **3**:211.
14. Dawson, P. J., A. H. Fieldsteel, and W. L. Bostick. 1963. Pathologic studies of Friend virus leukemia and the development of a transplantable tumor in BALB/c mice. *Cancer Res.* **23**:349.
15. Fieldsteel, A. H., P. J. Dawson, and J. Scholler. 1966. Virus-free Friend virus-induced tumor: *in vitro* and *in vivo* characteristics. *J. Natl. Cancer Inst.* **36**:71.
16. Ostertag, W., H. Melderis, G. Steinheider, N. Kluge, and S. Dube. 1972. Synthesis of mouse haemoglobin and globin mRNA in leukaemic cell culture. *Nat. New Biol.* **239**:231.
17. Friend, C., W. Scher, J. G. Holland, and T. Sato. 1971. Hemoglobin synthesis in murine virus-induced leukemic cells *in vitro:* stimulation of erythroid differentiation by dimethyl sulfoxide. *Proc. Soc. Natl. Acad. Sci. U. S. A.* **68**:378.
18. Fieldsteel, A. H., C. Kurahara, and P. J. Dawson. 1969. Friend virus-induced reticulum cell sarcomas grown *in vitro:* further evidence for the absence of Friend virus. *Cancer Res.* **29**:1846.
19. Dawson, P. J., R. B. Tacke, and A. H. Fieldsteel. 1968. Relationship between Friend virus and an associated lymphatic leukemia virus. *Br. J. Cancer.* **22**:569.
20. Steeves, R. A. 1975. Spleen focus-forming virus in Friend and Rauscher leukemia virus preparations. *J. Natl. Cancer Inst.* **54**:289.
21. Fieldsteel, H. A., P. J. Dawson, and C. Kurahara. 1971. *In vivo* and *in vitro* recovery of defective Friend virus by various leukemia viruses. *Int. J. Cancer.* **8**:304.
22. Lala, P. K. 1974. Changes in antigenicity of the Ehrlich ascites tumor by propagation in different host strains. *Proc. Am. Assoc. Cancer Res.* **15**:117. (abstr.)
23. Steeves, R. A., E. A. Mirand, S. Thomson, and L. Avila. 1969. Enhancement of spleen focus formation and virus replication in Friend virus-infected mice. *Cancer Res.* **29**:1111.