

# Helper-Dependent Properties of Friend Spleen Focus-Forming Virus: Effect of the *Fv-1* Gene on the Late Stages in Virus Synthesis

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Co-infection of neonatal BALB/c mice with Friend virus (FV) complex (containing defective spleen focus-forming virus [SFFV] and endogenous N-tropic leukemia-inducing helper virus [LLV-F]) and B-tropic Tennant leukemia virus (TenLV) resulted in the inhibition of LLV-F by the *Fv-1<sup>b</sup>* gene and recovery of a TenLV pseudotype of SFFV, abbreviated SFFV(TenLV). The host range of this pseudotype was B-tropic, since SFFV(TenLV) was 10 to 100 times more infectious for B-type (*Fv-1<sup>bb</sup>*) than for N-type (*Fv-1<sup>nn</sup>*) mice. The similar patterns of neutralization of N-tropic and B-tropic SFFV by type-specific murine antisera suggested that the difference in infectivity between these two SFFV preparations did not reside in envelope determinants. Rather, helper control of SFFV's host range was only apparent and dependent upon the ability of associated virus to provide a helper function for late stages in SFFV synthesis. Early stages in SFFV's infectious cycle were shown to be helper independent. The *Fv-1* gene did not act at the level of the cell membrane to effectively restrict SFFV infection, since SFFV-induced transformed cells could be detected in the absence of spleen focus formation and SFFV synthesis. Further, the generation of these transformed cells by SFFV followed a one-hit, dose-response pattern, suggesting that SFFV-induced cell transformation is helper independent. Finally, restriction of helper function by *Fv-1* may be an intracellular event, because both SFFV and its associated LLV-F helper share common envelope determinants and presumably adsorb onto and penetrate target cells with equal efficiency.

Study of the relationship between the two distinct viruses which comprise the Friend virus (FV) complex has been advanced by the development of a quantitative in vivo assay for Friend spleen focus-forming virus (SFFV) (2) and by the development of a rapid in vivo assay for detecting murine leukemia virus (MuLV) helper activity for Friend SFFV (28). The ability of FV complex to rapidly induce erythroleukemia is thought to be a property of SFFV which is accompanied by a lymphatic leukemia-inducing helper virus (LLV-F) in the FV complex (27). It has now been established that SFFV is defective, i.e., dependent upon its associated MuLV helper for focus formation in vivo (6), and that all known MuLV strains provide a helper function for defective SFFV. Further, SFFV acquires the type-specific envelope of its associated helper virus (7).

It would now appear that the nature of SFFV defectiveness and the mechanism of action of the *Fv-1* gene are related problems since restric-

tion of MuLV helper by *Fv-1* also restricts SFFV-induced focus formation. The *Fv-1<sup>b</sup>* gene of B-type mice (13, 18, 19, 22) interferes with MuLV having an N-tropic host range (11). Thus, infection of adult BALB/c mice (*Fv-1<sup>bb</sup>*) with FV complex containing SFFV and N-tropic LLV-F helper facilitated the demonstration of SFFV dependence upon LLV-F for focus formation. Titration of N-tropic FV complex in BALB/c mice yielded a multiple-hit, dose-response curve, whereas addition of excess MuLV helper converted the titration pattern to a one-hit form (6). The *Fv-1* gene functions in vitro to block MuLV synthesis and may exert regulatory control upon the ability of induced C-type virus (N-tropic) to persist in BALB/c cells after chemical activation (21, 29). These findings, together with those of Ware and Axelrad (34), suggest that the conferral of resistance to MuLV infection by the *Fv-1* gene operates at the cellular level and is a host response specifically related to the tropism of the infecting

virus. Studies in vivo have shown that the *Fv-1<sup>b</sup>* and *Fv-1<sup>n</sup>* genes inhibit the expression of N- or B-tropic MuLV helpers, respectively (Eckner and Styles, unpublished data), but it is not known whether this locus controls the adsorption and penetration of MuLV as well as SFFV into erythropoietic target cells, or the ability of the SFFV and MuLV genomes to replicate within infected cells, or both. The data presented here indicate that the expressed helper-dependence of SFFV for focus formation is in fact a reflection of SFFV dependence upon its associated helper virus (LLV-F) for late stages in virus synthesis. The *Fv-1* gene does not act at the level of the cell membrane to restrict SFFV infection since SFFV-induced transformed cells can be detected in the absence of virus-induced spleen foci and recovery of infectious SFFV. These transformed cells carry the SFFV genome which can be rescued upon addition of excess MuLV helper. This suggests that SFFV is able to transform mouse erythropoietic target cells into transplantable tumor colony-forming units (TCFU) without requiring MuLV helper. Further, it appears that restriction of MuLV helper by *Fv-1* is an intracellular event, because both SFFV and its associated helper share common envelope determinants and therefore presumably adsorb onto and penetrate target cells with equal efficiency. In the absence of productive MuLV infection, however, SFFV is not able to complete its infectious cycle, i.e., acquire a type-specific envelope and be released from the infected target cell.

## MATERIALS AND METHODS

**Mice.** Male and female 6- to 8-week-old DBA/2 (substrains Ha, Cr, and J), BALB/cCr, AKR, C3H/He, C57BL/6, and female Ha/ICR (random-bred) Swiss mice were obtained from the West Seneca Animal Production Unit, Roswell Park Memorial Institute, Buffalo, N.Y. In addition, WB, 129, I/St, C58/J, and C57BL/10 mice were obtained from T. S. Hauschka of the Institute. Female NZW mice were kindly provided by G. Cudkovic, State University of New York at Buffalo, and hybrid mice were bred in this laboratory.

**Viruses.** SFFV, as contained in the Mirand strain (16) of the FV complex, was originally obtained from Charlotte Friend and has now undergone over 200 cell-free passages in Ha/ICR Swiss mice. All such SFFV stocks initiate infection 50 to 100 times more efficiently in Swiss and DBA/2 mice than in BALB/c mice (26) and are therefore designated as having an N-tropic host range (11).

In an attempt to prepare a B-tropic pseudotype of Friend SFFV, a leukemia-inducing virus isolated by Tennant (31), referred to here as TenLV, was mixed with N-tropic FV complex and injected into B-type mice. TenLV was chosen because it is a B-tropic virus and shows high levels of helper activity for defective

Friend SFFV (Eckner, unpublished data). The details of pseudotype production are given below.

TenLV was obtained from R. A. Steeves, Albert Einstein College of Medicine, N.Y. Both TenLV and NB-tropic LLV-F stocks were maintained in this laboratory by serial passage of 20% (wt/vol) cell-free extracts of leukemic tissue into newborn BALB/c mice and were stored at  $-196^{\circ}\text{C}$ .

**Virus titrations.** All SFFV preparations were titrated in vivo with the spleen focus assay method (2). Briefly, 0.5-ml samples of diluted SFFV were injected intravenously into susceptible mice. Nine days later, their spleens were removed and fixed in Bouin solution. Discrete foci on the splenic surface were counted by macroscopy, and virus titers (mean number of foci per spleen  $\times$  dilution factor) were expressed in focus-forming units (FFU) per milliliter, where one FFU is the amount of virus required to induce an average of one focus per spleen.

All TenLV and LLV-F stocks were free of detectable SFFV and had demonstrated helper activity for SFFV. The origin of LLV-F and quantification of MuLV with a helper virus assay method have been described (28). Briefly, a constant amount of FV complex containing helper-dependent "indicator" SFFV was added either to potential helper virus, serially diluted from 1:10 to 1:320 in phosphate-buffered saline (PBS), or to PBS alone. Samples (0.5 ml) were then injected into the lateral tail vein of male BALB/c mice, seven per group. After 9 days the mice were killed, their spleens were fixed in Bouin fluid, and the number of discrete foci on the splenic surface  $>0.5$  mm in diameter were counted by macroscopy. The mean number of foci per spleen for each group, when multiplied by the SFFV dilution factor, gave an estimate of the titer of SFFV with or without diluted helper virus. The difference between these two estimates (i.e., the helper virus activity in  $\Delta$  FFU per milliliter) was then related on a log/log plot to the dilution factor of the helper virus. The linear extrapolation for the helper activity of undiluted virus is expressed in helper units (HU), where one HU is that amount of virus required to increase the estimated titer of SFFV by 1 FFU/ml.

**Pseudotype production.** A TenLV pseudotype of Friend SFFV was prepared in vivo as previously described (7) and is referred to here as SFFV(TenLV). Results obtained using SFFV(TenLV) prepared by F. Lilly (Albert Einstein College of Medicine, N.Y.) were identical to those obtained by using a pseudotype prepared in this laboratory. Briefly, SFFV (as contained in N-tropic FV complex) was diluted 1:100 into B-tropic TenLV helper so that the mixture contained  $10^2$  FFU/ml of SFFV (as titrated in DBA/2 mice) and  $10^3$  HU/ml of TenLV. Young (5 to 10 days old) BALB/c mice were inoculated intraperitoneally with 0.1 ml of this mixture, and 14 to 21 days later plasma from each animal was pooled and immediately used for a second passage or stored at  $-196^{\circ}\text{C}$ . The same protocol was followed for a second and third passage in 5- to 10-day-old BALB/c mice to reduce the relative concentration of N-tropic LLV-F indigenous to the FV complex. Subsequent passages of SFFV(TenLV) were conducted with adult BALB/c mice since this proved to be a routinely high titer virus preparation stable with respect to host range markers.

**Spleen colony assay for SFFV-induced tumor cells.** SFFV-induced tumor cells were assayed on the basis of spleen colony formation in heavily irradiated (800 rads) syngeneic mice (32). Spleen foci cannot be induced by SFFV in these animals, but normal hemopoietic colonies are detected. To distinguish between SFFV-induced tumor colonies and normal hemopoietic colonies, cell suspensions prepared from the spleens of FV-infected BALB/c mice were incubated with either normal mouse serum (NMS) or specific Friend cytotoxic antiserum (as a final serum dilution of 1:10) and complement (guinea pig complement, Grand Island Biological Co., Grand Island, N.Y.) in vitro at 37 C for 30 min. This Friend antiserum had no cytotoxic effect for normal colony-forming cells. After incubation, the cell suspensions were diluted and injected into groups of 10 X-irradiated BALB/c mice (800 rads). Ten days later, the mice were sacrificed and their spleens were removed and fixed in Bouin solution. The mean number of normal colonies observed per spleen, when subtracted from the mean number of colonies observed after incubation of a sample from this same spleen cell suspension with normal mouse serum, gave an estimate of the number of SFFV-induced tumor cells (expressed as TCFU) recovered per spleen. This value, when multiplied by the original SFFV dilution factor, provided an estimate of the number of TCFU generated per milliliter of stock SFFV. One TCFU is defined as a cell or group of cells acting as a unit which after intravenous injection reaches the spleen and gives rise by proliferation to one tumor colony, and which loses tumor colony-forming ability after incubation with cytotoxic Friend antiserum.

**Preparation of murine antisera.** Antiserum directed against either Moloney leukemia virus (MoLV) or Rich leukemia virus (RichLV) was prepared from adult BALB/c mice given 20 weekly intraperitoneal injections of  $10^8$  freeze-thawed BALB/c lymphoma cells induced by Moloney leukemia virus and Rich leukemia virus, respectively. In addition, Friend LL and SimL antisera were prepared from adult Swiss mice given 16 immunizations of  $10^8$  Swiss lymphoma cells induced by LLV-F or SimLV, respectively. SimLV refers to a leukemia-inducing virus isolated in this laboratory from spontaneous lymphomas of SIM/McK mice. Swiss mice injected by the same schedule with normal Ha/ICR Swiss (random-bred) spleen cells did not produce alloantibodies in quantities sufficient to neutralize SFFV. Within each series of immunizations, the mice were bled 7 days after the last injection. The sera were pooled, heated at 56 C for 30 min to eliminate complement activity, and stored at -70 C. All sera used demonstrated virus-neutralizing activity against the MuLV pseudotype of SFFV corresponding to the MuLV-induced lymphoma cells used as pretreatment materials. In addition, Friend LL antiserum contained cytotoxic antibodies reactive with both LLV- and SFFV-induced tumor cells. Also, these activities were completely removed by three serial adsorptions with appropriate MuLV-induced lymphoma cell homogenates. As a control, NMS was obtained from an untreated group of Swiss mice.

**Virus neutralization.** The antigenicity of all SFFV

preparations was tested by following their neutralization kinetics in vitro with specific antisera (25). Antiserum (final dilution 1:10 or 1:20) was prewarmed to 37 C, and at time zero a given SFFV preparation ( $10^3$  to  $5 \times 10^4$  FFU/ml) was combined with the diluted serum and incubated in a 37 C water bath. At 10-min intervals over a 60-min period, a sample was removed from the reaction tube, diluted in ice-cold PBS (pH 7.2), and immediately assayed in groups of seven susceptible mice by the spleen focus assay method. Since SFFV(LLV-F) is expressed maximally in N-type mice and SFFV(TenLV) is most readily detected in B-type mice, assays to detect surviving SFFV(LLV-F) and SFFV(TenLV) were conducted in DBA/2Ha and BALB/c mice, respectively. The fractional virus survival for each incubation period was calculated by dividing the corresponding residual virus titer by the original virus titer (SFFV incubated with NMS for 60 min at 37 C). Serum potencies are expressed in terms of the inactivation constant  $K$ , determined as follows:  $K = (D/t) \log_e(V_0/V_t)$ , where  $D$  = serum dilution,  $V_0$  = original virus titer, and  $V_t$  = virus titer at reaction time  $t$ .

## RESULTS

**Altered host range of SFFV progeny after mixed infection of BALB/c mice with N-tropic FV complex and B-tropic TenLV helper.** Hartley et al. (11) have found that the MuLV can be classified into one of three host range categories called *tropisms*. N-tropic viruses initiate infection more efficiently on NIH Swiss (N-type) than on B-type (BALB/c) mouse embryo cells, whereas B-tropic viruses show the reciprocal pattern. Continued passage of an MuLV in the restrictive host cell type results in the recovery of virus that infects both cell types with equal efficiency. Such virus is designated NB-tropic.

In a previous study (26), it was demonstrated that the host range of N-tropic FV complex (containing defective SFFV and LLV-F helper) could be converted to an NB-tropism after a minimum of three serial passages in newborn BALB/c mice. The altered infectivity of SFFV was due, at least in part, to a change in the ability of LLV-F to express its helper function in B-type mice. However, whether the host range of defective SFFV is a helper-controlled property remained an unanswered question, because SFFV itself could have also adapted to productively infect B-type mice. Therefore, to determine whether SFFV host range is a helper-controlled property, SFFV(TenLV) was prepared. Strict control of SFFV host range by its associated TenLV helper would result in the appearance of B-tropic SFFV, whereas altered host range due to SFFV host adaptation would result in the appearance of NB-tropic SFFV. New SFFV stocks were prepared by co-infecting

BALB/c mice with helper-dependent SFFV and TenLV helper (see Materials and Methods). The host range of this new SFFV preparation was determined quantitatively by observing the dose-response relationships of SFFV(TenLV) in BALB/c mice as well as in four different strains of N-type (*Fv-1<sup>nn</sup>*) mice. The estimated virus titer in FFU per milliliter was constant at all virus dilutions over a 20-fold range in BALB/c mice (Fig. 1). This one-hit, dose-response pattern was due to a direct proportionality between spleen focus counts and virus dose at all dilutions for which there were countable spleens. In contrast, maximal titer estimates in N-type mice (DBA/2, AKR, and C3H) were usually from 10- to 100-fold lower, and the dose-response relationships of SFFV(TenLV) observed in DBA/2Cr and C3H were multiple-hit and two-hit, respectively. Although both DBA/2Ha and AKR mice were relatively resistant to SFFV(TenLV), the dose-response relationships were very near a one-hit pattern. This may be due to genes other than *Fv-1<sup>n</sup>* conferring resistance to either TenLV or SFFV, or both.

To determine more fully the host range of SFFV(TenLV), this virus was titrated in several other strains of N- and B-type mice, as well as in *Fv-1<sup>nb</sup>* hybrids. For purposes of comparison, N-tropic FV complex [SFFV(LLV-F)] was titrated in these same strains of mice. SFFV(TenLV) showed a pattern of infectivity reciprocal to that of N-tropic SFFV(LLV-F), and mice carrying both the *Fv-1<sup>n</sup>* and *Fv-1<sup>b</sup>* alleles were relatively resistant to both viruses, indicating that the *Fv-1* alleles are dominant for resistance to N- and B-tropic viruses in vivo (Table 1). Both N- and B-type mice (C58/J, B6, and B10), homozygous for resistance at *Fv-2* (13), completely blocked spleen focus formation by these strains of SFFV. Finally, it should be pointed out that AKR mice, although *Fv-1<sup>nn</sup>* and capable of restricting the function of B-tropic MuLV in vitro, are very resistant to both SFFV(LLV-F) (Table 1 and reference 4) and SFFV(TenLV), and show one-hit, dose-response patterns for both viruses. Resistance to focus formation by N-tropic FV complex again suggests that AKR mice carry genes conferring resistance to SFFV, since AKR mice would not be expected to restrict N-tropic LLV-F helper in vivo.

The host range of SFFV(TenLV) is typical of a B-tropic virus, and mixed infection of BALB/c mice with SFFV(LLV-F) and TenLV may have resulted in the appearance of progeny SFFV which carry B-tropic TenLV host range determinants in their envelope. However, an alternative explanation is that SFFV can infect both

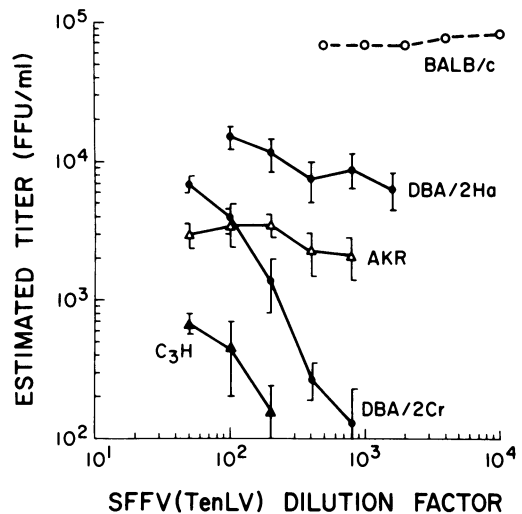


FIG. 1. Dose-response relationships of B-tropic Friend spleen focus-forming virus, SFFV (TenLV), as titrated in N-type (*Fv-1<sup>n</sup>*) mice. The broken line represents a typical titration of SFFV (TenLV) in B-type (*Fv-1<sup>b</sup>*) mice.

N- and B-type mice with equal efficiency in the presence or absence of MuLV helper, and that SFFV is dependent upon helper virus for the synthesis of infectious progeny with resultant induction of spleen foci. Thus, SFFV helper-dependence for host range control would be only apparent and based on SFFV inability to complete its entire infectious cycle in the absence of actively replicating MuLV helper. This matter is considered in detail in the following sections.

**Defectiveness of Friend SFFV for the synthesis of infectious progeny.** The observation that Friend SFFV is defective for focus formation in vivo (6) and that SFFV acquires the type-specific envelope antigen(s) of its associated helper virus (7) suggested that the participation of helper virus in the infectious cycle of SFFV is essential for SFFV maturation. In the present study, therefore, I tested the ability of SFFV to generate new infectious progeny either in the presence or absence of large amounts of co-infecting helper virus. To quantitate the appearance of infectious SFFV in the plasma of mice infected with several different SFFV doses, plasma from all mice was collected 9 days after infection, pooled within each group, and assayed for the presence of infectious N-tropic or B-tropic SFFV in susceptible DBA/2Ha, or BALB/c mice, respectively, with the spleen focus assay method (Materials and Methods). The mean number of FFU of SFFV contained per milliliter of plasma, when multiplied by the original SFFV dilution

TABLE 1. Effect of N-tropic (LLV-F) and B-tropic (TenLV) helpers on the expressed host range of Friend SFFV

Host <sup>a</sup>	Estimated virus titer <sup>b</sup> (Mean ± SE)			
	SFFV(LLV-F)	Titration pattern	SFFV(TenLV)	Titration pattern
<b>N-type (<i>Fv-1<sup>na</sup></i>)</b>				
Ha/ICR Swiss	33,000 ± 10,000	1-hit	3,700 ± 980	2-hit
DBA/2Ha	28,000 ± 750	1-hit	14,200 ± 1,800	>1-hit
DBA/2Cr	46,000 ± 6,900	1-hit	7,200 ± 1,000	>2-hit
DBA/2J	47,000 ± 4,600	1-hit	5,700 ± 1,000	>2-hit
WB	42,000 ± 5,000	1-hit	2,600 ± 1,100	2-hit
C3H/He	13,600 ± 3,900	1-hit	700 ± 100	2-hit
AKR	430 ± 78	1-hit	360 ± 90	1-hit
129	47,000 ± 11,000	1-hit	240 ± 100	>2-hit
NZW	15,000 ± 3,000	1-hit	3,400 ± 400	2-hit
C58/J	0		0	
<b>B-type (<i>Fv-1<sup>bb</sup></i>)</b>				
BALB/cCr	500 ± 90	>2-hit	63,000 ± 8,800	1-hit
I/St	790 ± 200	>2-hit	30,000 ± 2,200	1-hit
A/He	200 ± 60	2-hit	15,000 ± 3,000	1-hit
C57BL/6 (B6)	0		0	
C57BL/10 (B10)	0		0	
<b>NB-type (<i>Fv-1<sup>nb</sup></i>)</b>				
(B10 × DBA)F <sub>1</sub>	600 ± 100	2-hit	10,000 ± 1,900	2-hit
(C3H × B10)F <sub>1</sub>	2,500 ± 500	2-hit	2,700 ± 680	2-hit
(B10 × WB)F <sub>1</sub>	850 ± 90	2-hit	9,400 ± 1,400	2-hit

<sup>a</sup> All male and female 6- to 8-week old mice of a given strain responded identically to virus infection.

<sup>b</sup> Estimated titers are expressed in focus-forming units per milliliter and represent the highest value obtained when each SFFV preparation was assayed at several dilutions, similar to those presented in Fig. 1. This was necessary because SFFV(LLV-F) showed multiple-hit titration patterns in B- and NB-type mice, whereas SFFV(TenLV) showed multiple-hit titration patterns in B- and NB-type mice.

factor, gave an estimate of the amount of infectious SFFV generated per milliliter of stock SFFV over a 9-day period. The dose-response relationship for the appearance of infectious SFFV in the plasma of BALB/c mice infected with N-tropic SFFV(LLV-F) was multiple-hit (Fig. 2A). In contrast, the appearance of infectious SFFV in the plasma of DBA/2Ha mice infected with N-tropic SFFV(LLV-F) or in the plasma of BALB/c mice infected with N-tropic SFFV(LLV-F) and an excess of NB-tropic LLV-F helper showed a one-hit, dose-response pattern. These results indicate that inhibition of the natural N-tropic helper virus in the FV complex by the *Fv-1<sup>b</sup>* gene of B-type mice (i.e., functional elimination of LLV-F at high dilutions of FV complex) drastically reduced the ability of SFFV to generate infectious progeny. Similar results were obtained when B-tropic SFFV(TenLV) was assayed for the generation of infectious SFFV in the presence and absence of excess NB-tropic helper virus (Fig. 2B). The *Fv-1<sup>a</sup>* gene effectively reduced the level of functional B-tropic TenLV helper in DBA/2Ha mice and resulted in the observed multiple-hit, dose-response titration pattern for

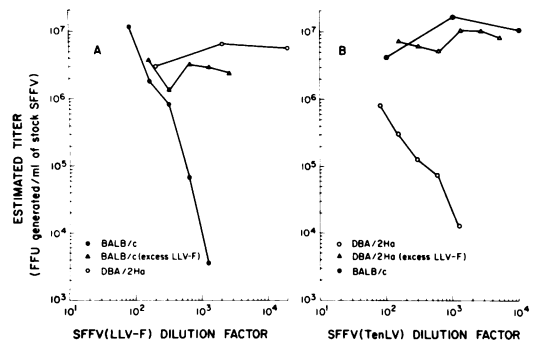


FIG. 2. Dose-response relationships for the appearance of infectious SFFV in the plasma of mice infected with either (A) N-tropic SFFV (LLV-F) with and without excess ( $10^3$  helper units) NB-tropic LLV-F in the diluent, or (B) B-tropic SFFV (TenLV) with and without excess ( $10^3$  helper units) NB-tropic LLV-F in the diluent. Standard errors were from 10 to 20% of the means.

SFFV(TenLV). As with the synthesis of infectious SFFV(LLV-F) progeny in BALB/c mice, there was a  $10^3$ -fold increase in the amount of infectious SFFV recovered at high virus dilu-

tions when SFFV(TenLV) was grown in the presence of excess NB-tropic LLV-F helper.

To determine whether the synthesis of infectious SFFV is dependent upon the relative dose of NB-tropic LLV-F helper, helper virus assays were designed to detect LLV-F helper activity for both direct focus formation by SFFV and the synthesis of mature SFFV progeny. Before termination of the helper assay as described in Materials and Methods and presented in Fig. 3 (lower line), plasma from all mice was collected, pooled within each group, and assayed in susceptible DBA/2Ha mice for the presence of SFFV. The mean number of foci per spleen for each group, when multiplied by the plasma dilution factor, gave an estimate of the titer of SFFV(LLV-F) recovered per milliliter of plasma after growth in the presence or absence of diluted helper virus. The difference between these two estimates represents the helper activity in  $\Delta$  FFU of infectious SFFV recovered per milliliter of plasma. The upper line of Fig. 3 shows the results of this experiment. The slope of the regression line is  $-0.61$  and the Y-intercept is  $19.4 \times 10^4$  HU. This represents an 8.3-fold increase in the level of helper activity expressed by LLV-F when compared with the values obtained by using a direct focus assay (Fig. 3, lower line). The slope of this regression line is  $-0.45$ , and  $Y = 23.5 \times 10^3$  HU. These results demonstrate that the amount of new

infectious SFFV in the plasma of infected mice is related to the amount of functional helper virus available.

#### Fv-1 control of cellular resistance to N- and B-tropic pseudotypes of Friend SFFV.

From the experiments just described, it can be seen that the presence of excess NB-tropic helper virus totally obscures the expression of both the *Fv-1<sup>b</sup>* and *Fv-1<sup>n</sup>* alleles which are dominant for resistance to the N-tropic MuLV helper found in the SFFV-LLV-F complex and to the B-tropic helper found in the SFFV-TenLV complex, respectively. Although this genetic resistance can be overcome, the mechanism by which *Fv-1* restricts MuLV helper function, and thus SFFV, is unknown. A genetic block to MuLV infection may occur at the step of viral adsorption or penetration into a potential target cell, or it may be the result of intracellular events such as viral nucleic acid restriction. The results of experiments designed to determine whether N-tropic SFFV(LLV-F) could adsorb onto N- or B-type spleen cells *in vitro* indicated that SFFV is NB tropic with respect to its ability to adsorb onto these target cells. When 200 FFU of N-tropic FV complex were mixed with either  $10^8$  DBA/2Ha or  $10^8$  BALB/c spleen cells (diethylaminoethyl-dextran treated) and allowed to interact with these cells for 30 min at 37 C, less than 10% (12-18 FFU) of the infectious virus was recovered in each case. Appropriate controls showed that this phenomenon was not the result of nonspecific loss of infectious SFFV, and a detailed report of this study will appear elsewhere. In the present study, however, it was possible to determine whether SFFV could penetrate erythropoietic target cells *in vivo* in the absence of "functioning" MuLV helper. BALB/c mice were infected with 300 FFU of FV complex containing SFFV and N-tropic LLV-F. Inhibition of endogenous LLV-F by the *Fv-1<sup>b</sup>* gene greatly restricted SFFV expression (Table 2). However, simultaneous administration of a high dose of NB-tropic LLV-F ( $10^3$  HU) to BALB/c mice as well as infection with helper 1 or 3 days after infection with N-tropic SFFV(LLV-F) resulted in increased SFFV titer estimates for both the induction of spleen foci and the generation of infectious progeny. Similar results were obtained when DBA/2Ha mice were given 100 FFU of FV complex containing SFFV and B-tropic TenLV. The *Fv-1<sup>n</sup>* gene functionally eliminated TenLV helper to the extent that an average of only 3.0 foci were induced per spleen. Addition of exogenous NB-tropic LLV-F helper on day 0, 1, or 3 resulted in a  $>10$ -fold increase in the average number of foci induced per spleen and a  $>10^2$ -fold increase in the synthesis of infectious

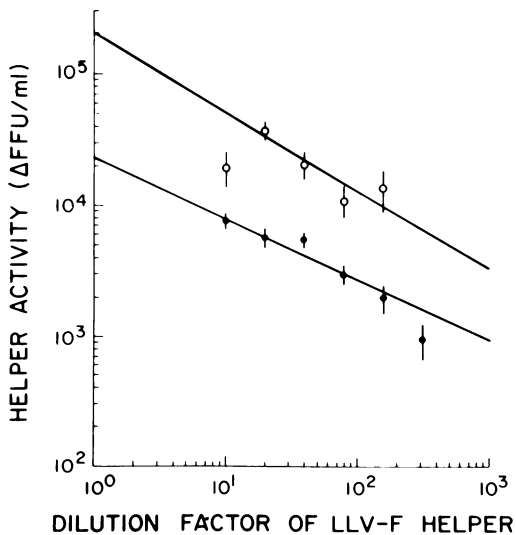


FIG. 3. Relationship between helper activity (mean  $\pm$  SE) and the dilution factor of NB-tropic LLV-F added to a stock of defective SFFV(LLV-F) indicator. Symbols: ●, helper activity expressed in  $\Delta$  FFU per milliliter for direct focus formation; O, helper activity expressed in  $\Delta$  FFU of infectious SFFV recovered per milliliter of plasma.

TABLE 2. Focus formation and synthesis of infectious SFFV progeny in N- and B-type mice infected with FV complex only, co-infected with NB-tropic LLV-F helper, or given LLV-F at various times after SFFV infection

FV complex <sup>a</sup>	Host	Helper virus <sup>b</sup> (interval)	No. of foci per spleen	No. of SFFV progeny recovered (FFU/ml of plasma) <sup>c</sup>
N-tropic [SFFV(LLV-F)]	BALB/c	None	15.8	10 <sup>3</sup>
		LLV-F (0 day)	85.1	160 × 10 <sup>3</sup>
		LLV-F (1 day)	56.2	100 × 10 <sup>3</sup>
		LLV-F (3 days)	30.0	60 × 10 <sup>3</sup>
B-tropic [SFFV(TenLV)]	DBA/2Ha	None	3.0	0.12 × 10 <sup>2</sup>
		LLV-F (0 day)	41.3	41.0 × 10 <sup>2</sup>
		LLV-F (1 day)	36.0	26.0 × 10 <sup>2</sup>
		LLV-F (3 days)	46.5	15.0 × 10 <sup>2</sup>

<sup>a</sup> FV complex (0.5 ml per animal) containing either 3 × 10<sup>3</sup> FFU of SFFV and N-tropic LLV-F or 10<sup>2</sup> FFU of SFFV and B-tropic TenLV was injected intravenously on day 0 into BALB/c or DBA/2 mice, respectively.

<sup>b</sup> Each animal received 10<sup>3</sup> helper units of NB-tropic LLV-F intravenously together with SFFV, or 1 or 3 days after SFFV infection.

<sup>c</sup> Because SFFV(LLV-F) is expressed maximally in N-type mice and SFFV(TenLV) is most readily detected in B-type mice, assays for the presence of infectious SFFV(LLV-F) and SFFV(TenLV) in the plasma of infected mice were performed in DBA/2Ha and BALB/c, respectively.

SFFV progeny on days 1 and 3. The ability to rescue SFFV as long as 3 days after infection of a restrictive host indicates that SFFV can, in the absence of functional helper, infect both N- and B-type mice. This suggests that the *Fv-1* gene, though capable of restricting MuLV helper function, does not effectively restrict SFFV penetration into erythropoietic target cells. Further evidence supporting this conclusion comes from the observation that SFFV-transformed cells can be recovered from the spleens of infected BALB/c mice (see Materials and Methods) in the absence of detectable focus formation and synthesis of infectious virus. The dose-response pattern for the production of transplantable Friend TCFU (Fig. 4) indicates that this is not a helper-dependent event. The numbers of TCFU produced in the 9-day period after infection of adult BALB/c mice with serial dilutions of N-tropic FV complex are directly proportional to the SFFV dose and appear to be independent of the presence of LLV-F helper. Addition of excess helper resulted in an upward shift of the dose-response curve (4). However, the dose-response pattern remained one-hit. This shift was most likely due to increased production of infectious SFFV with secondary infection of adjacent target cells. In contrast to the one-hit pattern observed for the generation of TCFU by N-tropic FV complex, both the synthesis of infectious virus and induction of spleen foci by SFFV followed two-hit, dose-response patterns (Fig. 4). A potential complication of this *in vivo* assay for Friend TCFU is that the cell preparations to be assayed by infusion into lethally irradiated recipient

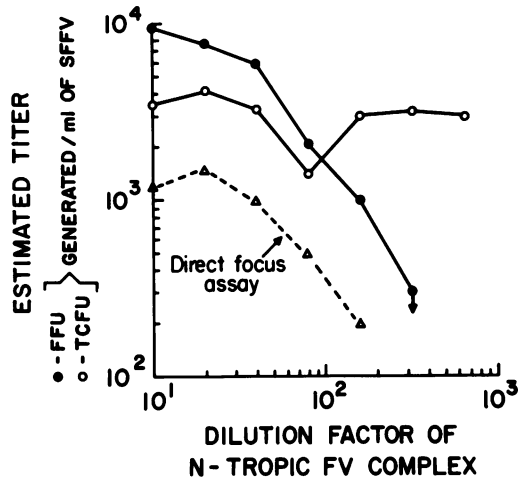


FIG. 4. Dose-response relationships for the appearance of infectious SFFV in the plasma (●) and SFFV-induced tumor colony-forming units (TCFU) in the spleens (○) of BALB/c mice infected with FV complex containing SFFV and N-tropic LLV-F. The broken line represents a titration of this same SFFV (LLV-F) preparation in BALB/c mice conducted in parallel with the virus and TCFU recovery study.

BALB/c mice contain normal erythroid cells as well as TCFU and virus. These normal cells are potential targets for SFFV infection, and increased availability of targets at high cell doses (infused into recipient mice) could result in exaggerated TCFU estimates where sufficient SFFV is also in the inoculum. Therefore, the observed curve would tend towards a two-hit pattern as a function of infection of target cells in the inocula containing the highest concentra-

tions of infectious SFFV. However, the very fact that the observed curve is one-hit indicates that this feature of the assay does not affect the results and that SFFV can transform erythropoietic target cells in the absence of functioning helper virus since TCFU were detected at limiting dilutions of FV complex (1:320 and 1:640) in the absence of detectable helper-dependent SFFV synthesis and focus formation.

**Analysis of the type-specific envelope antigens of SFFV(TenLV).** Because the only known contribution of helper virus to SFFV is envelope protein and because genetic cellular resistance appears to be directed against N- and B-tropic MuLV helper function and apparently not against defective SFFV directly, it became important to determine whether TenLV contains a unique antigen(s) in its envelope which confers B-tropism to this helper virus and is available for incorporation into the envelope of SFFV. Based on the work of Vogt, who described in detail the role of envelope antigens in the avian tumor group (33), it is reasonable to assume that the envelope of TenLV plays a decisive role in determining its B-tropic host range. Why, then, does SFFV which is grown in the presence of TenLV helper and presumably acquires the TenLV type-specific envelope not itself acquire a strict B-tropic host range for early stages in virus synthesis (i.e., adsorption and penetration)? In an attempt to partially answer this question, the antigenicity of SFFV(LLV-F) and SFFV(TenLV) was tested by observing the kinetics of neutralization of each SFFV preparation in vitro by specific Friend, Rich, and Moloney typing antisera. Friend and Rich typing antisera effectively neutralized SFFV(LLV-F), whereas Moloney antiserum had no neutralizing activity for this same preparation of Friend SFFV (Fig. 5A). Similarly, SFFV prepared from BALB/c mice after passage in the presence of TenLV helper, SFFV(TenLV), was strongly neutralized by both Friend and Rich antisera but not by Moloney antiserum (Fig. 5B). Clearly, use of these typing sera failed to demonstrate the presence of a *new* antigen or antigenic grouping in the envelope of SFFV(TenLV). A fourth typing antiserum directed against a leukemia virus isolated from SIM/McK mice (SimLV) and capable of strongly neutralizing SFFV(LLV-F) ( $K = 1.0$ ) also neutralized the TenLV pseudotype of SFFV ( $K = 1.15$ ). Neutralization of each SFFV pseudotype was not due to neutralization of its homologous helper virus since the addition of excess helper virus to susceptible mice infected with neutralized SFFV did not result in increased focus forma-

tion. Preliminary results with Tennant antiserum prepared from BALB/c mice immunized with TenLV-induced BALB/c lymphoma cells indicate that the envelope of SFFV(TenLV) does not contain neutralizable antigen(s) not found in SFFV(LLV-F). This virus-neutralizing antiserum is a low-titer preparation, and further analysis of these viruses with high-titer Tennant antiserum and antiserum directed against other B-tropic MuLV will be necessary to confirm the antigenic identity reported here. However, the similar patterns of SFFV(LLV-F) and SFFV(TenLV) neutralization by the four type-specific murine antisera demonstrates that the difference in infectivity between these two SFFV preparations does not reside in SFFV envelope antigen. Rather, this difference is most likely dependent upon the ability of associated helper virus to contribute helper function to SFFV for late stages in virus synthesis and maturation.

## DISCUSSION

An analysis of the defectiveness of Friend SFFV for focus formation in vivo has not only increased our knowledge of the relationship between SFFV and its associated helper virus LLV-F, but has also contributed in part to our understanding of the mechanism of action of the *Fv-1* gene. For the first time, it is possible to consider in some detail the infectious cycle of Friend SFFV, the role of its associated MuLV helper, and the genetic mechanisms by which a host may restrict productive SFFV infection. A schematic representation of the infectious cycle of Friend SFFV is presented in Fig. 6 and represents the combined efforts of many investigators. First, let us consider what I refer to as the "helper-independent" properties of Friend

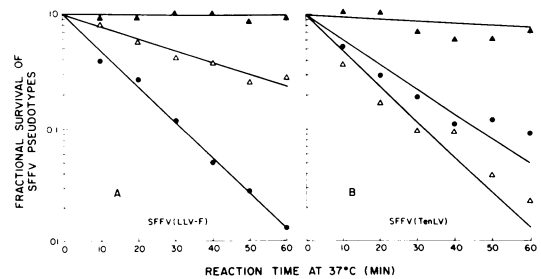


FIG. 5. Neutralization kinetics of (A) SFFV (LLV-F) and (B) SFFV-(TenLV), incubated with type-specific murine antisera. Symbols for A: ●, Friend LL antiserum,  $K = 1.40 \pm 0.03$ ; △, Rich antiserum,  $K = 0.23 \pm 0.02$ ; ▲, Moloney antiserum,  $K = 0.0$ . Symbols for B: ●, Friend LL antiserum,  $K = 0.90 \pm 0.09$ ; △, Rich antiserum,  $K = 0.65 \pm 0.04$ ; ▲, Moloney antiserum,  $K = 0.09 \pm 0.03$ .



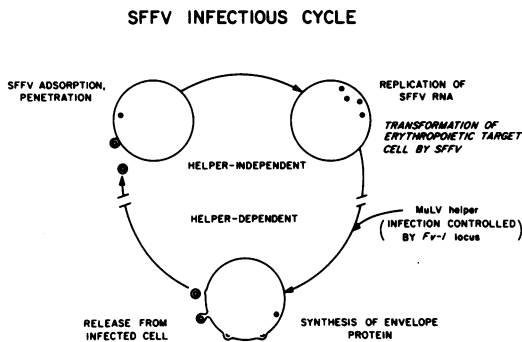


FIG. 6. Schematic representation of the dichotomy of the infectious cycle of FV complex containing defective SFFV and an MuLV helper (LLV-F). SFFV is helper-dependent for the acquisition of its type-specific envelope and therefore for release from an infected cell. The restriction of MuLV helper function by the *Fv-1* locus is most likely an intracellular event and, more correctly stated, is a restriction of productive MuLV infection.

SFFV. It would appear from the data presented in Table 2 and Fig. 4 and from the results of experiments designed to determine whether N-tropic SFFV could adsorb onto N- and B-type spleen cells in vitro (Eckner, unpublished data) that SFFV is NB-tropic with respect to its ability to adsorb onto and penetrate erythropoietic target cells. I have made the assumption that the envelope of SFFV is identical to that of its associated MuLV helper. This is a reasonable assumption since analysis of the neutralizable envelope antigens of SFFV (MuLV) pseudotypes indicates that SFFV is antigenically indistinguishable from its helper (7). If this assumption is correct, then N-tropic as well as B-tropic MuLV helpers also adsorb onto and penetrate N- and B-type target cells with equal efficiency since these events are dependent upon cell membrane-viral envelope interaction. This calls for the obvious suggestion that the *Fv-1* gene operates not at the level of the cell membrane, but restricts productive MuLV infection via nucleic acid restriction or other intracellular events. This would then represent a mechanism of genetic resistance different from that reported for the avian tumor virus system. Genetic resistance of chicken embryo cells to infection by the Bryan strain of Rous sarcoma virus is controlled by a single pair of autosomal genes (23), and this resistance occurs at the step of viral penetration of the cell membrane (20).

It appears that SFFV can not only enter but also replicate its genome within an infected cell in the absence of MuLV helper. Fieldsteel et al.

(8, 9) have demonstrated that FV-induced reticulum cell sarcomas, after extended growth in vitro, were free of infectious SFFV and MuLV helper based on the absence of budding type-C particles in these cultures and based on the inability to induce typical Friend disease (erythroleukemia) by using cell-free fluids from these cultures. The SFFV genome was present, however, since SFFV rescue from this cell culture was routinely accomplished after the addition of LLV-F or other MuLV helpers. Data presented here suggest that cell transformation is also a helper-independent property of SFFV. However, recent experiments indicate that some neoplastic properties of the Friend cell may in some fashion be dependent upon the cell's ability to elaborate infectious virus.

From the above discussion, it would appear that defective Friend SFFV is in many ways similar to murine sarcoma virus (MSV). Considerable evidence now exists that MSV can adsorb onto and enter a mouse embryo cell without requiring MuLV helper (12), and that MSV is able to transform mouse cells in the absence of co-infecting MuLV (1, 3, 24). However, MuLV helper is required for the replication of infectious MSV with resultant focus formation via spread of infection, and possibly is required for the induction of new surface antigen in the transformed target cell (30). It is not known whether infection by SFFV alone results in the appearance of new cell surface antigen; however, it does follow that the apparent defectiveness of SFFV for focus formation in vivo is primarily a reflection of SFFV dependence upon its associated helper virus for rapid synthesis of infectious SFFV progeny. A spleen cell infected with both SFFV and MuLV produces progeny resulting in rapid enlargement of the focus by spread of SFFV infection. A cell infected by SFFV only, although transformed, might be expected to form a focus more slowly and not be initially detected, since the focus must enlarge by cell division alone. Thus, the "helper-dependent" properties of SFFV include the acquisition of a type-specific envelope, release from the infected cell, and focus formation in vivo. Because restriction of MuLV helper by the *Fv-1* gene results in decreased synthesis of mature SFFV and decreased SFFV-induced focus formation, the apparent host range of SFFV is also a helper-controlled property. However, this host range most correctly refers to the expression of SFFV infection and not to the early stages of infection.

Clearly, there are many questions that remain unanswered concerning the relationship between Friend SFFV and its MuLV helper.

SFFV may be deficient in ribonucleic acid (RNA)-dependent deoxyribonucleic acid (DNA) polymerase activity as has been described for Rous sarcoma virus and MSV (10, 17, 15), or there may be differences between the RNA of SFFV and nontransforming murine viruses with respect to size and subunits (5, 14, 29), or both. A thorough knowledge of the complementation of functions between SFFV and MuLV helper within a common host may one day allow us to develop the means of combating some of the lethal effects of these viruses.

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