A CLASSIFICATION OF THE MURINE LEUKEMIA VIRUSES

NEUTRALIZATION OF PSEUDOTYPES OF FRIEND Spleen Focus-Forming Virus by Type-Specific Murine Antisera*

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It is still debatable whether the various isolates of murine leukemia-inducing viruses (MuLV) represent distinct viruses, isolates from different sources of the same virus, or closely related variants. The studies of Gross (1), designed to compare these viruses in terms of their morphology, chemical stability, and pathogenicity, failed to reveal significant differences. Electron microscopic observations have shown that all MuLV isolates are enveloped, "C-type" particles (1, 2). In addition, these particles are uniformly susceptible to mild ether treatment (1, 3), share a common internal group-specific antigen (4, 5), and are capable of inducing a wide spectrum of leukemias, namely lymphoid, myeloid, or stem cell leukemias among different susceptible mouse strains (1, 6). Only the MuLV strains isolated by Friend (7) and Rauscher (8) consistently induce a particular type of leukemia, an erythroleukemia, in all susceptible mouse strains. The capacity of these virus isolates to rapidly induce erythroleukemia is thought to be a property of spleen focus-forming virus (SFFV), which is regularly accompanied by a lymphatic leukemia-inducing helper virus (LLV-F or LLV-R) in the Friend or Rauscher virus complex, respectively (9).

Methods used for the classification of leukemia viruses of avian origin have been based on their host range in genetically different types of chicken cells, and their patterns of interference among one another. Results from these tests were then confirmed by virus neutralization tests with specific antisera (10). While leukemia viruses

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Abbreviations used in this paper: FFU, focus-forming units; FMR antigen(s), Friend-Moloney-Rauscher antigen(s); FV, Friend virus complex; G antigen(s), Gross antigen(s); HU, helper units; K, serum inactivation constant; LCM, lymphocytic choriomeningitis; LLV-F and LLV-R, Friend (Rauscher) lymphatic leukemia-inducing virus; MuLV, murine leukemia virus; NMS, normal mouse serum; PBS, phosphate-buffered saline; RV, Rauscher virus complex; SFFV, spleen focus-forming virus. Other virus isolates with their abbreviations are listed in Table I.
of murine origin exhibit both host-range differences and viral interference patterns, such parameters cannot be utilized for classifying MuLV's. For example, naturally occurring MuLV strains fall into two categories with respect to their growth in mouse embryo (ME) cells of NIH Swiss (N-type) or BALB/c (B-type) mice (11). "N-tropic" viruses initiate infection more efficiently on N-type than on B-type ME cells, while "B-tropic" viruses show the reciprocal pattern. However, these host-range patterns are not stable, since continued passage of an MuLV strain 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" (11).

Interference tests are complicated by a lack of specificity, for such unrelated agents as an arbovirus (12) and a parainfluenza virus (13) inhibit MuLV replication and leukemia induction in vivo. Therefore, a classification for MuLV isolates depends on an immunologic approach. Studies by Old et al. (14, 15) have revealed two broad categories of virus-induced murine leukemias, those possessing the Gross or G antigen(s), and those possessing the Friend-Moloney-Rauscher or FMR antigen(s). However, the more subtle antigenic relationships among the leukemia viruses that induce tumors of G or FMR specificity remained largely unknown.

To classify the MuLV's according to their type-specific (virus-bound), envelope antigens, we wished to apply the technique of antigenic analysis by neutralization kinetics, a method previously used to study the antigenic relationship between Friend SFFV and Rauscher SFFV (16). However, the spleen focus assay method (17) used in this study could not be directly applied to MuLV's other than SFFV. Until the recent work of Rowe et al. (18), an enumerative assay for MuLV's other than SFFV was not available. Therefore, we utilized the observation that the \textit{Fv-P} gene of B-type mice (19, 20) interferes with LLV-F, the naturally occurring helper virus for SFFV in the Friend virus (FV) complex. Preliminary results (21) indicated that mixed infection of neonatal BALB/c mice with FV complex (containing SFFV and N-tropic LLV-F) and an antigenically distinct MuLV resulted in antigenic alteration of SFFV. We refer to such virus preparations as pseudotypes of Friend SFFV, abbreviated SFFV(MuLV) following the example of Hanafusa et al. (22) with Rous sarcoma virus. The experiments reported here confirm the observation that SFFV(MuLV) pseudotypes can be prepared in vivo. Further, the patterns by which a given SFFV(MuLV) pseudotype was neutralized by specific antisera identified the antigenicity of the corresponding MuLV used to prepare the pseudotype. Accumulated data made it possible to classify several MuLV's according to their virus-bound, type-specific envelope antigens.

\textit{Materials and Methods}

\textit{Animals.}—Neonatal BALB/c and C3H/He mice and 6-8-wk old male BALB/cCr, DBA/2Ha, and female Ha/ICR (random-bred) Swiss mice were obtained from the West Seneca Animal Production Unit, Roswell Park Memorial Institute, Buffalo, N.Y. Female Sprague-Dawley rats were obtained from the Charles River Breeding Laboratories, Inc., Wilmington, Mass.

\textit{Viruses.}—SFFV, as contained in the Mirand strain (23) of the FV complex, was originally
obtained from Dr. Charlotte Friend and has now undergone over 200 cell-free passages in Ha/ICR Swiss mice. All such SFFV stocks initiate infection 40–100 times more efficiently in Ha/ICR Swiss and DBA/2 mice than in BALB/c mice (24) and are therefore designated as having an N-tropic (11) host range. BALB/c mice and other B-type mice are significantly less sensitive to N-tropic viruses. The multiple-hit, dose-response relationships observed when SFFV is titrated in BALB/c mice (24) suggests that B-type mice inhibit the N-tropic helper virus (LLV-F) indigenous to the FV complex (9).

SFFV, as contained in the Rauscher virus (RV) complex, was originally obtained from Dr. F. Rauscher, and has now undergone 10 cell-free passages in adult Ha/ICR Swiss mice. This RV complex, although originally NB-tropic, now initiates spleen focus formation 50 times more efficiently in DBA/2 mice than in BALB/c mice and shows a two-hit, dose-response relation-

TABLE I

Identification and Origin of Viruses Used to Prepare Pseudotypes of SFFV

| MuLV isolate* | Origin (reference) | Method used to remove SFFV | Titer helper units (HU)/ml |
|---------------|--------------------|---------------------------|---------------------------|
| LLV-F‡        | Friend (7, 9)      | C57BL passage             | 50,000                    |
| LLV-R         | Rauscher (8, 9)    | Sucrose gradient          | 20,000                    |
| RPV (= LLV-F) | Rowson and Parr (27, 28) | End point dilution       | 15,000                    |
| RichLV        | Rich et al. (29)   | Free of detectable SFFV when isolated | 25,000 |
| MolLV         | Moloney (30)       |                           | 30,000                    |
| BMLV          | Breyere et al. (31)|                           | 5000                      |
| SimLV         | Spontaneous (21)   |                           | 20,000                    |
| 334C          | Buffett et al. (32)|                           | 14,000                    |
| GLV           | Gross (33)         |                           | 30,000                    |
| GilLV         | Graffi (34)        |                           | 10,000                    |
| Other murine viruses |            |                           |                           |
| MPV           | Molomut-Padnos (26)|                          |                           |
| LCM           | Traub (35)         |                           |                           |

* Most MuLV isolates were maintained in B-type mice; all MuLV’s demonstrated an NB-tropic host range before use in pseudotype production.
† This virus is referred to in the text as either LLV-F or simply LLV.

ship when titrated in BALB/c mice (R. J. Eckner, unpublished observations). Apparently the natural helper virus (LLV-R) in the RV complex acquired an N-tropic host range after prolonged passage in Swiss mice in our laboratory.

In an attempt to prepare pseudotypes of Friend SFFV, several MuLV and other viruses of murine origin were mixed with N-tropic FV complex and injected into B-type mice. The virus preparations used are listed in Table I with their titers in helper units per milliliter (see section on Virus Titrations). All MuLV stocks were maintained in this laboratory by serial passage of 20% (w/v) cell-free extracts of leukemic tissue into newborn BALB/c mice and were stored at −196°C. Lymphocytic choriomeningitis (LCM) virus, a known arenavirus (25), was chosen as a nonlethal, nonleukemogenic “control” murine virus, as was Molomut-Padnos virus (MPV), a leukopenia-inducing, RNA-containing virus (26) that may be related to LCM virus, but is still unclassified. Both virus preparations had titers >10⁷ ID₅₀/ml in adult Swiss mice, and were kindly provided by Dr. J. Hotchin (Virus Laboratory, State of New York Department of
Health, Albany, N.Y.) and Dr. M. Padnos (Waldemar Medical Research Foundation, Inc., Long Island, N.Y.), respectively.

Virus Titrations.—SFFV in the FV or the RV complex was titrated in vivo with the spleen focus assay method (17). Briefly, 0.5-ml samples of diluted SFFV were injected intravenously into susceptible DBA/2 mice. 9 days later, their spleens were removed and fixed in Bouin’s solution. Discrete foci on the splenic surface were counted macroscopically, and virus titers (mean No. of foci per spleen × dilution factor) were expressed in focus-forming units (FFU)/ml, where one FFU is that amount of virus required to induce an average of one focus per spleen.

All other MuLV stocks were free of detectable SFFV and had demonstrable helper activity for SFFV. Their origin and quantification with a helper virus assay method have been described (36). Briefly, a constant amount of FV complex containing helper-dependent “indicator” SFFV was added either to potential helper virus, serially diluted from 1:5 to 1:40 in phosphate-buffered saline (PBS), or to PBS alone. Samples of 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’s fluid, and the numbers of discrete foci on the splenic surface >0.5 mm in diameter were counted macroscopically. 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 AFFU/ml) 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 one FFU/ml.

Pseudotype Production.—MuLV pseudotypes of Friend SFFV were prepared in vivo as summarized in Fig. 1. Briefly, SFFV (as contained in N-tropic FV complex) was diluted 1:100 into various NB-tropic MuLV helper stocks, so that the mixture contained 10³ FFU/ml of SFFV (as titrated in DBA/2 mice) and 10³ HU/ml of MuLV. We inoculated 5-10-day old BALB/c mice intraperitoneally with 0.2 ml of this mixture, and 20-25 days later the enlarged spleens were pooled, from which cell suspensions (20% w/v) were made in McCoy’s medium with 3% fetal calf serum. The suspensions were centrifuged at 2400 g for 10 min, and the supernatant fluids (referred to as spleen extracts) were either used immediately for a second passage or stored at −196 °C. The same protocol was followed with each SFFV(MuLV) preparation for a total of two to three passages in order to reduce the relative concentration of LLC-F indigenous to the FV complex.

Preparation of Murine Antisera.—Moloney, Rich, Breyere-Moloney, and Graffi antisera were prepared from adult BALB/c mice given 20 weekly intraperitoneal injections of 10⁸ frozen-thawed BALB/c lymphoma cells induced by MolLV, RichLV, BMLV, and GilLV, respectively. In addition, Friend LL and SimL antisera were prepared from adult Swiss mice given 16 immunizations of 10⁷ Swiss lymphoma cells induced by LLV-F or SimLV, respectively. Swiss mice injected by the same schedule with normal Ha/ICR Swiss 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 material. Also, this activity was completely removed by three serial absorptions with appropriate MuLV-induced lymphoma cell homogenates. As a control, normal mouse serum (NMS) was prepared from an untreated group of Swiss mice.

Gross Typing Serum.—In an attempt to prepare specific neutralizing antiserum directed against Gross virus, Sprague-Dawley rats were immunized with 5 × 10⁸ Gross virus-induced BALB/c lymphoma cells (spleen and thymus). The first injection (1.5 ml intraperitoneally)
included 0.5 ml of complete Freund's adjuvant. The second, third, and fourth injections (0.5 ml intramuscularly) were given on days 10, 25, and 40, respectively. 7 days after the last treatment, the animals were bled and sera were prepared as stated above. Finally, the Gross antiserum was serially absorbed 12 times with normal BALB/c spleen cells and was tested for its neutralizing capacity against different pseudotypes of SFFV.

**Virus Neutralization.**—The antigenicitiy of all SFFV preparations was tested by following their neutralization kinetics in vitro with specific antisera (16). Antiserum (final dilution 1:10 or 1:20) was prewarmed to 37°C, and at time zero a given SFFV preparation (10⁶ to 5 × 10⁷ 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 DBA/2 mice by the spleen focus assay method. 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: $K = (D/d)\log (V_o/V_t)$, where $D = serum$ dilution, $V_o = original$ virus titer, and $V_t = virus$ titer at reaction time $t$.

**RESULTS**

**Scheme for Producing MuLV Pseudotypes of SFFV.**—It has been shown that all N-tropic strains of FV complex tested are defective for spleen focus formation in B-type mice (37). This defectiveness of SFFV, together with the observations that (a) SFFV's antigenicity appears to be controlled by its associated helper virus (21) and (b) most MuLV preparations express significant levels of helper activity for SFFV, suggested that MuLV pseudotypes of SFFV could be prepared in vivo. Fig. 1 summarizes a proposed scheme for preparing pseudotypes in neonatal BALB/c mice. In this example, SFFV and N-tropic LLV (as contained in FV complex) are mixed with a preparation of NB-tropic MolLV. The proposed pseudotype recovered, SFFV(MolLV), has both the antigenicity and the host range of the MolLV helper virus.

The validity of this pseudotype hypothesis is dependent upon the demonstration that: (a) all MuLV preparations used can replicate efficiently (i.e. they can replicate within 20 days of infection, to titers above the input virus inoculum so that MuLV envelope antigens would be available in B-type mice for pseudotype production); (b) coinfection of mice with SFFV and antigenically distinct MuLV helpers results in an antigenic alteration of SFFV; (c) the antigenic alteration is not merely apparent, due to nonspecific virus interactions within a common host, but is a specific change that occurs only when LLV-F is inhibited in mice coinfected with an antigenically distinct helper virus.

**Replication of MuLV Helper Viruses in Neonatal BALB/c Mice.**—Studies concerning the growth of LLV-F in adult BALB/c mice (9) suggested that MuLV helpers could only replicate transiently in adult mice, with subsequent loss of demonstrable virus beyond 14 days postinfection. Therefore, before attempting to produce pseudotypes of SFFV, we had to determine if MuLV preparations could replicate efficiently in neonatal BALB/c mice for a more prolonged period of time. To follow the growth kinetics of several MuLV
stocks (candidates for pseudotype production), we inoculated neonatal (5-10-day old) BALB/c mice intraperitoneally with MolLV (3 × 10^3 helper units [HU]/animal), or LLV-R (10^6 HU), or GLV (3 × 10^3 HU). At various times after infection, groups of three to four mice were sacrificed, the enlarged spleens were pooled, and cell suspensions (1 spleen equivalent/ml medium) were prepared in McCoy's medium with 3% fetal calf serum. The suspensions were centrifuged at 2400 g for 15 min and the supernatant fluids were assayed for helper activity for SFFV. As shown in Fig. 2, peak titers for MolLV, LLV-R, and GLV were observed from 14 to 21 days postinfection, and diminishing

![Diagram](image.png)

**Fig. 1. Scheme for producing MuLV pseudotypes of Friend SFFV.** Helper-dependent SFFV is diluted 1:10 into stock helper virus (>10^6 HU) and 0.2 ml of this mixture (200 FFU, as titrated in DBA/2 mice) is injected intraperitoneally into 5-10-day old BALB/c mice. Pseudotype virus is harvested 20-25 days postinfection from the spleens of infected mice.

titers were observed for MolLV and GLV from 18 to 35 days postinfection. Similarly, titers >10^6 HU/spleen were recovered from BALB/c mice 2-3 wk postinfection at 5-10 days of age with LLV-F, RichLV, RPV, GiLV, SimLV, BMLV, and 334C virus. Therefore, the readily detectable helper activity for SFFV seen with all MuLV stocks tested suggests that they can replicate efficiently (as defined above) in neonatal BALB/c mice.

**Efficiency and Stability of SFFV Antigenic Alteration.**—In a preliminary study (21) we demonstrated that the antigenicity of SFFV can be altered by serially passaging a mixture of SFFV and an antigenically distinct helper virus (334C) in newborn BALB/c mice. In the present study, the efficiency of SFFV antigenic alteration was determined quantitatively after each of three serial
passages with MoLV in neonatal BALB/c mice. MoLV was chosen because of its efficient helper activity for SFFV, and because preliminary studies with Moloney antiserum suggested that MoLV was antigenically distinct from SFFV(LLV).

New SFFV stocks were prepared by coinfected 5–10-day-old BALB/c mice with helper-dependent SFFV and MoLV helper (Fig. 1). At each passage level, the antigenicity of the new SFFV preparation was tested by observing the kinetics of neutralization of SFFV in vitro by specific Friend or Moloney typing antiserum. As shown in Table II, Friend typing antiserum neutralized the source material for these experiments, SFFV(LLV), while Moloney antiserum had no neutralizing activity for this same preparation of Friend SFFV. In contrast, SFFV prepared from neonatal BALB/c mice after one passage in the presence of MoLV (P1:MoLV) was neutralized by both Friend and Moloney antisera. This result suggests the presence of two antigenically distinct SFFV populations, or the existence of SFFV particles with a mosaic of LLV-F and MoLV antigens in their envelope (38). Finally, SFFV prepared after two and three passages in the presence of MoLV (P2:MoLV and P3:MoLV, respectively) was effectively neutralized only by the Moloney antiserum.

These observations indicate that mixed infection of BALB/c mice with SFFV(LLV) and MoLV results in the appearance of a pseudotype of SFFV which carries the Moloney type-specific antigen(s) in its envelope. The complete loss of antigen detected by the Friend typing antiserum suggests (a) that N-tropic LLV-F indigenous to the FV complex has been functionally eliminated.
after two passages with MolLV and (b) that the envelope of the pseudotype virus, SFFV(MolLV), is homogeneous, i.e., it does not contain antigen(s) characteristic of both Friend and Moloney virus.

We also examined the stability of SFFV antigenic alteration, for this alteration might represent a genetic change, such as recombination (39) between SFFV and MolLV helper. Recombinants should be relatively stable with respect to their envelope antigens. We tested this possibility by analyzing the neutralizable antigens of new SFFV stocks prepared from neonatal BALB/c mice infected with SFFV(MolLV) only, or from BALB/c mice infected with

### TABLE II

**Efficiency and Stability of SFFV Antigenic Alteration**

| Infecting viruses | Virus harvested passage No.:helper virus | Neutralization by specific typing antisera (K ± sd)* | Friend | Moloney |
|-------------------|-----------------------------------------|----------------------------------------------------|--------|---------|
| SFFV(LLV-F)       | P0:LLV                                  | 1.37 ± 0.07                                        | 0      |         |
| P0:LLV + MolLV    | P1:MolLV                                | 1.66 ± 0.12                                        | 0.28 ± 0.04 |
| P1:MolLV + MolLV  | P2:MolLV†                               | 0                                                  | 1.21 ± 0.05 |
| P2:MolLV + MolLV  | P3:MolLV‡                               | 0                                                  | 1.13 ± 0.04 |

SFFV(MolLV) passaged in BALB/c mice with no helper virus added

|                   |                                           |                                                   |        |         |
|---                |                                           |                                                   |        |         |
| P2:MolLV only     | P1:-- only                               | 0                                                  | 0.23 ± 0.09 |
| P1:-- only        | P2:-- only                               | 0                                                  | 0.47 ± 0.05 |
| P2:-- only        | P3:-- only                               | 0                                                  | 1.09 ± 0.09 |

SFFV(MolLV) passaged in BALB/c mice with LLV-F added

|                   |                                           |                                                   |        |         |
|---                |                                           |                                                   |        |         |
| P2:MolLV + LLV-F  | P1:LLV-F                                | 1.27 ± 0.12                                        | 0      |         |
| P1:LLV + LLV-F    | P2:LLV-F                                | 1.41 ± 0.10                                        | 0      |         |

* Virus-neutralizing activity is expressed in terms of the inactivation constant $K$. Each $K$ value was obtained by following the neutralization kinetics of SFFV preparations by specific antiserum at 10-min intervals for a total of 60 min.

† These SFFV preparations are identified as MolLV pseudotypes and abbreviated SFFV-(MolLV).

both SFFV(MolLV) and $10^6$ HU of NB-tropic LLV-F. In the absence of continued mixed infection of BALB/c mice with SFFV(MolLV) and MolLV (middle section of Table II), pseudotype virus was antigenically stable. Only Moloney antiserum neutralized these SFFV preparations. Both first and second passage viruses (P1:-- and P2:--) were low in titer and were prepared as 50% (rather than 20%) spleen extracts. An excess of viral antigen in these preparations might explain the apparently decreased $K$ values observed. SFFV(MolLV) prepared as a 20% spleen extract after a third passage with no MolLV helper added also proved to be antigenically stable. However, this antigenic stability was conditional, since SFFV(MolLV) was easily converted back to the LLV-F type (lowest part of Table II) after only one passage in BALB/c mice with
NB-tropic LLV-F (P1:LLV-F). The antigenic stability of SFFV(MolLV) may be the result of substitution by MolLV for LLV-F in the FV complex, since NB-tropic MolLV replicates efficiently in BALB/c mice, as demonstrated in Fig. 2, and N-tropic LLV-F is functionally eliminated. The antigenic interconversion between LLV-F and MolLV types demonstrates that SFFV is defective with respect to the expression of neutralizable envelope antigens, but this does not rule out the possible existence of a minor fraction of recombinants not detectable in our system.

Requirements for the Production of SFFV Pseudotypes.—The possibility

| TABLE III |
|------------------|------------------|------------------|------------------|
| Requirements for Alteration of SFFV Envelope Antigens |
| Helper virus | Neutralization of SFFV by specific antisera (K ± st)* |
| Helper virus | Friend | Moloney | MPV§ |
| SFFV(LLV-F) passed in vivo with | | |
| No helper added | ~ | ~ | 1.30 ± 0.07 | 0 | 0 |
| LLV-F | None | 80,000 | 1.92 ± 0.18 | 0 | 0 |
| MolLV | None | 30,000 | 0 | 1.01 ± 0.07 | NT |
| MolLV Heat inactivated at 56°C for 60 min | 0 | 1.70 ± 0.07 | 0 | NT |
| MPV | None | 0 | 1.64 ± 0.03 | NT | 0 |
| SFFV(LLV-F) mixed in vitro with | | |
| MolLV Incubated with SFFV at 37°C for 30 min | 30,000 | 1.45 ± 0.10 | 0 | NT |
| MolLV Pelleted with SFFV at 100,000 g for 90 min | 35,000 | 1.19 ± 0.06 | 0 | NT |

NT = not tested.

* Virus-neutralizing activity is expressed in terms of the inactivation constant K. Each K value was obtained by following the neutralization kinetics of SFFV preparations by specific antiserum at 10-min intervals for a total of 60 min.

§ MPV antiserum, provided by Dr. M. Padnos, was prepared from a rabbit immunized with HeLa cell-grown MPV and has a virus neutralization titer >10³.

 existed that SFFV antigenic alteration could have resulted from incorporation into the SFFV virion of helper MuLV envelope antigen that was present in the cell as a result of the initial infectious event (i.e., antigen that did not require MuLV synthesis). To test this possibility, we passaged SFFV serially in BALB/c mice which were also injected with heat-inactivated MolLV (56°C for 60 min). After two such passages, conversion to the Moloney type was not observed (Table III). As a control, SFFV(LLV) was grown with an NB-tropic strain of LLV-F. This SFFV preparation was also neutralized only by Friend LL antiserum. In agreement with previous data, SFFV was readily converted to the Moloney pseudotype when the MolLV preparation used was infective. This indicates that the acquisition of distinct new envelope antigens is dependent upon the replicative capacity of MolLV.
To determine if SFFV pseudotypes could be prepared by using murine viruses other than leukemia viruses, SFFV stocks were also prepared after two serial passages in BALB/c mice with either MPV or LCM virus. Although MPV is known to interfere with Friend and Rauscher SFFV-induced splenomegaly and spleen focus formation (R. J. Eckner, unpublished observations; and personal communication from Dr. M. Padnos), we were able to recover SFFV from neonatal BALB/c mice coinfected with MPV. This SFFV preparation was antigenically indistinguishable from the source material, SFFV(LLV). Both were strongly neutralized by Friend LL antiserum, while MPV antiserum failed to neutralize either SFFV preparation (Table III). Attempts to grow SFFV with LCM virus in BALB/c mice were not successful, as LCM virus interfered strongly with SFFV.

| Infecting viruses* | Host (genotype) | Neutralization by specific typing antisera ($K \pm SE$) |  |
|-------------------|----------------|---------------------------------|---|
| N-tropic SFFV(LLV-F) + MoLV | C3H/He ($Fv-1^a$, $Fv-1^a$) | 2.14 ± 0.40 | 0.33 ± 0.07 |
| NB-tropic SFFV(LLV-F) + MoLV | BALB/c ($Fv-1^b$, $Fv-1^b$) | >2.0 | 0.12 ± 0.06 |
| N-tropic SFFV(LLV-F) + MoLV | BALB/c ($Fv-1^a$, $Fv-1^a$) | 0 | 1.20 ± 0.05 |

* All in vivo growth of SFFV comprised two serial passages with $10^4$ HU of NB-tropic MolLV.

† Virus-neutralizing activity is expressed in terms of the inactivation constant $K$. Each $K$ value was obtained by following the neutralization kinetics of SFFV preparations by specific antisera at 20-min intervals for a total of 60 min.

The possibility of antigenic alteration due to interviral aggregates between SFFV and MolLV was also tested. SFFV was either incubated with $>10^4$ HU of MolLV in vitro for 30 min at 37°C, or centrifuged with MolLV at 105,000 g for 90 min (Spinco rotor No. 40; Spinco Division, Beckman Instruments, Inc., Fullerton, Calif.) and resuspended to the original SFFV volume. These procedures failed to demonstrate any conversion of SFFV(LLV) to the Moloney type (lower section of Table III).

Finally, it is implied in the pseudotype hypothesis (Fig. 1) that pseudotype production would require inhibition of the natural helper virus in the FV complex, LLV-F. For the $Fv-1$ gene to effect this inhibition, the $b$ allele should be expressed and the FV complex should be N-tropic. It was possible to test this requirement for pseudotype production by analyzing the neutralizable envelope antigens of SFFV stocks prepared from: (a) neonatal C3H/He mice ($Fv-1^a$, $Fv-1^a$) infected with N-tropic FV complex and $10^4$ HU of NB-tropic MolLV;
(b) neonatal BALB/c mice (Fv-P, Fv-P) infected with BALB-adapted (NB-tropic) FV complex and 10⁶ HU of NB-tropic MolLV; or (c) neonatal BALB/c mice infected with N-tropic FV complex and 10⁴ HU of NB-tropic MolLV. In the first case (a), the Fv-1 b gene is absent, while in the second (b), Fv-1 b should

be unable to confer resistance to LLV-F since the helper has an NB-tropic host range. Virus neutralization tests with specific Friend or Moloney typing antisera confirmed this prediction (Table IV), for SFFV(LLV-F) was effectively converted to the Moloney type only when the Fv-1 P allele was present and able to confer resistance to endogenous N-tropic LLV-F of the FV complex.
**MuLV Type-Specific Envelope Classification.**—With the requirements for and efficiency of pseudotype production established, nine different SFFV(MuLV) pseudotypes were prepared. We then analyzed the neutralizable antigen(s) of each pseudotype by observing the kinetics of neutralization with a panel of type-specific antisera. The patterns by which a given SFFV(MuLV) pseudotype was neutralized allowed us to identify the virus-bound (type-specific) envelope antigens of the particular MuLV used. This technique is presented in Figs. 3 and 4, which demonstrate that LLV-F and MolLV pseudotypes are antigenically distinct. SFFV(LL-F) was effectively neutralized by Friend

| MuLV pseudotype | Serum inactivation constant (K ± se)* |
|-----------------|-------------------------------------|
| SFFV(GLV)       | 0.52 ± 0.07                        |
| SFFV(LLF)       | 0.70 ± 0.02                        |
| SFFV(RPV)       | 0.41 ± 0.04                        |
| SFFV(GiLV)      | 0.12 ± 0.03                        |
| SFFV(SimLV)     | 0.24 ± 0.14                        |
| SFFV(MolLV)     | 0.12 ± 0.07                        |
| SFFV(LL-R)      | 0.67 ± 0.04                        |
| SFFV(BMLV)      | 0.08 ± 0.02                        |
| SFFV(RichLV)    | 0.25 ± 0.06                        |
| SFFV(334C)      | 0                                  |

* Virus-neutralizing activity is expressed in terms of the inactivation constant K. Each K value was obtained by following the neutralization kinetics of SFFV preparations by specific antiserum at 10-min intervals for a total of 60 min.

† In contrast to other antisera (murine), Gross antiserum was prepared from rats immunized against GLV-induced, BALB/c lymphoma cells. Serial absorption with normal BALB/c spleen cells resulted in greatly decreased K values (from >2.0 to <0.25) against SFFV(LLF) and other SFFV(MoLV) pseudotypes, while the K value against SFFV(GLV) decreased only fourfold.

§ Rauscher virus complex and SFFV(LL-R) gave identical results.

¶ This K value was calculated from the initial slope of the neutralization curve since the virus became resistant to further neutralization after 15 min.

LL and SimL antisera (Fig. 3), while SFFV(MolLV) was effectively neutralized only by Moloney and Rich antisera (Fig. 4). With this particular SFFV(MolLV) preparation, Friend LL antiserum showed some neutralizing activity (K = 0.3), but four other SFFV(MolLV) preparations tested were not neutralized by this same Friend LL antiserum (K = 0).

Further analysis of the neutralizable envelope antigens of these and other SFFV(MuLV) pseudotypes made possible a "type-specific" SFFV(MuLV) envelope classification. Table V contains the serum inactivation constants (K) obtained by reacting nine pseudotypes with seven different typing antisera. SFFV(GLV) is antigenically distinct from all other MuLV pseudotypes tested, while SFFV(LL-F), SFFV(RPV), SFFV(GiLV), and SFFV(SimLV)
show almost identical patterns of reactivity with these typing sera. These and other distinctions become more obvious when a plus-minus scheme is substituted for the observed $K$ values (Table VI). “Plus” (+) represents significant virus neutralization ($K > 0.1$), and “minus” (−) represents virus neutralization not significantly different from control reactions with NMS ($K < 0.1$) at the 0.1% confidence level. Two major categories may be identified which correspond to the G and FMR subgroups. In addition, the FMR subgroup can be divided into four types on the basis of distinct neutralization patterns.

| MuLV isolate used in the pseudotype | Subgroup | Type | I | II | III | IV | V | VI | VII |
|-------------------------------------|----------|------|---|----|-----|----|----|-----|-----|
| Gross passage A                     | G        | I    | + | −  | −   | −  | −  | −   | −   |
| Friend                              | FMR      | 2A   | − | +  | +   | +  | +  | −   | −   |
| Rowson-Parr                         |          |      |   | +  | +   | +  | −  | −   | −   |
| Graffi                              |          |      |   | +  | +   | +  | +  | −   | −   |
| SIM leukemia                        |          |      |   | +  | +   | +  | +  | −   | −   |
| Moloney                             | FMR      | 2B   | − | −  | −   | +  | +  | −   | −   |
| Rauscher                            | FMR      | 2C   | − | +  | +   | +  | +  | +   | +   |
| Breyere-Moloney                     |          |      |   | +  | +   | +  | +  | +   | +   |
| Rich                                |          |      |   | +  | +   | +  | +  | −   | −   |
| Buffett (334C)                      | FMR      | 2D   | − | +  | −   | −  | +  | −   | −   |

* This subgroup designation is based on the virus-induced cellular antigens of mouse leukemias (14, 15, 40).

† Roman numerals have been arbitrarily assigned to viral envelope antigens that were detected by Gross (I), Friend LL (II), SimL (III), Moloney (IV), Rich (V), Breyere-Moloney (VI), and Graffi (VII) antisera. These antigens appear to be antigenically distinct from one another, as shown above.

§ Since rats immunized with “normal” mouse spleen cells provided serum which neutralized SFFV(LLV-F), SFFV(GLV), and other SFFV(MuLV) preparations, the plus-minus scheme substituted for the $K$ values obtained using Gross antiserum prepared in rats (Table V) is based on the following. “Plus” (+) represents significant virus neutralization, and “minus” (−) represents virus neutralization not significantly different from control reactions with rat antiserum prepared against normal mouse spleen cells ($K < 0.25$).

MuLV's of type 2A express the hypothetical antigens II, III, and sometimes V (as detected by Friend LL, SimL, and Rich antisera, respectively), while MoLV of type 2B expresses only antigens IV and V (as detected by Moloney and Rich antisera). Type 2C contains MuLV's that express antigens II to V inclusive, and sometimes VI, while type 2D is characterized by antigens II and V only.

It appears that our typing sera are detecting different antigens and not different concentrations of the same antigen(s). Evidence for this view is provided in Table VI, for the identification of each antigen or antigenic grouping can be exclusive of detection of all other type specificities.
DISCUSSION

Analysis of the neutralization kinetics of several SFFV(MuLV) pseudotypes has enabled us to classify these viruses according to their type-specific envelope antigens. We have made the assumption that the classification observed here for SFFV(MuLV) pseudotypes can be extended in parallel to the relationships among the MuLV isolates used to prepare these pseudotypes. This seems to be a reasonable assumption, because each antiserum in our panel effectively neutralized its respective pseudotype of SFFV. However, final proof of the identity between our SFFV(MuLV) classification and the true MuLV classification will have to await a similar neutralization analysis of these MuLV's with a plaque assay in vitro.

Our method of preparing SFFV(MuLV) pseudotypes was based on the successful competition in B-type mice of an NB-tropic MuLV over LLV-F, the N-tropic helper virus for SFFV in the FV complex. Functional elimination of LLV-F was demonstrated by the antigenic stability of the SFFV(MolLV) pseudotype (Table II), and by the constant rate of neutralization (Fig. 4) of most pseudotypes to low fractional survival values. A more conventional method for preparing pseudotypes would be to “rescue” SFFV from a virus-negative but SFFV-transformed cell line by superinfection of the cells with a different MuLV. An approach like this has been tried with some success by Fieldsteel et al. (41, 42) but they have not described neutralization tests with the spleen focus assay method, and therefore we cannot be sure that they have actually obtained pseudotypes of SFFV.

Our observation that LLV-F (Friend) and MolLV, while antigenically distinct from one another, share different antigens with LLV-R (Rauscher), is in keeping with some of Fink’s earlier results based on in vivo protection and immunofluorescence (43). Rich et al. (29) also observed a broad cross-reactivity among Friend, Moloney, Rauscher, and Rich viruses, but they used a heterologous antiserum prepared in rabbits. It is now known that unabsorbed rabbit antisera directed against normal mouse spleen cells can “neutralize” SFFV (24), and, in view of this, it is remarkable that Huebner (44), with typing antisera from rats, was able to observe distinct neutralization patterns of the Gross, Friend, Moloney, and Rauscher pseudotypes of murine sarcoma virus. Levy et al. (45) have also demonstrated the usefulness of specific murine antiserum, and have shown that MolLV and GiLV are antigenically distinct.

In the present study, rats immunized with normal BALB/c spleen cells provided serum which neutralized SFFV(LLV-F) as well as the SFFV(GLV) pseudotype. This serum activity was removed only after extensive absorption with BALB/c spleen cells. Further, antiserum from rats immunized with GLV-induced BALB/c lymphoma cells neutralized both SFFV(LLV-F) and SFFV(GLV). However, extensive absorption with normal BALB/c spleen cells provided us with Gross antiserum which preferentially neutralized SFFV(GLV) (Table V).

On the basis of the findings (that LLV-F, MolLV, and LLV-R are anti-
genically distinct) it appears that the specificity observed by cytotoxic G vs. FMR antisera is different from that observed by neutralization kinetics. We therefore suggest that the specific antigens revealed by cytotoxic tests (14, 15) be referred to as subgroup specific, while the MuLV envelope antigens revealed by virus neutralization tests be referred to as type specific. This is in keeping with evidence from several laboratories (46–48) that the cellular antigen detected in the cytotoxicity reaction is distinct from virus-bound envelope antigens of the corresponding MuLV.

With respect to the cross-reactivity observed among those MuLV strains listed in types 2A through 2D, several points should be made: (a) LLV-F and RPV have a common origin and it is therefore not unexpected that these viruses are antigenically indistinguishable; (b) it is unusual that SimLV belongs to type 2A since most MuLV’s associated with spontaneous murine leukemias belong to type 1 (on the basis of the induction of G cellular antigen); (c) the antigenic similarity between LLV-F and GiLV may or may not be related to their common origin from Ehrlich ascites tumors; (d) the apparent antigenic identity between SFFV(LLV-F) and SFFV(LLV-R) previously reported (16) was not confirmed here, presumably due to the greater “resolving power” of neutralization kinetics when seven (instead of three) typing antisera were used; (e) within type 2C, both LLV-R and BMLV were derived from murine tumors, while RichLV was isolated from mice given extracted Friend virus RNA. This latter fact, together with the distinct antigenicity of RichLV, suggests that RichLV originated from the mice and not from the FV complex; (f) the 334C (Buffett) virus is similar to SimLV in that not all MuLV isolates from so-called “spontaneous” leukemias are of type 1 antigenicity. However, 334C virus does resemble GLV in that we were unable to obtain virus-neutralizing antibodies from immunized mice. In future studies we shall examine the type-specific envelope antigens of other known MuLV isolates such as Stansly virus and Mazurenko virus. It may also be feasible to prepare pseudotypes of Rauscher SFFV, since our stock of RV complex is now N-tropic and is helper dependent in B-type mice.

Recently it has been shown that helper activity for SFFV is associated with avian leukemia viruses (ALV) and with feline leukemia virus (FeLV) (49), and also with tissue extracts from leukemic humans (50). To determine if ALV, FeLV, and helper preparations from leukemic humans (HuLH) can be used to prepare pseudotypes of SFFV is an important area for future study which may increase our understanding of leukemogenesis in man. We are encouraged by recent work (51) that has demonstrated antigenic alteration of SFFV grown with HuLH preparations in B-type mice. These SFFV preparations are abbreviated SFFV(HuLH) and are neutralized by serum from patients whose spleens provided active HuLH extracts, but are not effectively neutralized by Friend typing antiserum.
SUMMARY

Coinfection of neonatal BALB/c mice with helper-dependent Friend spleen focus-forming virus (SFFV), as contained in the Friend virus (FV) complex, and antigenically distinct Moloney leukemia virus (MoLV) resulted in the recovery of a MoLV pseudotype of SFFV, abbreviated SFFV(MoLV). The antigenic alteration of SFFV was observed by following its neutralization kinetics in vitro by specific Friend or Moloney typing antiserum.

Effective pseudotype production was accomplished only when N-tropic LLV-F (the natural helper virus in the FV complex) was inhibited in B-type mice coinfected with an NB-tropic MoLV or other murine leukemia virus (MuLV) preparation. SFFV pseudotypes could not be prepared by using murine viruses other than leukemia viruses. SFFV prepared after two serial passages in the presence of MoLV was effectively neutralized by Moloney antiserum, but not by Friend typing antiserum; therefore, the envelope of the pseudotype virus, SFFV(MoLV), is homogeneous. Pseudotype virus was antigenically stable in the absence of continued mixed infection of BALB/c mice with SFFV(MoLV) and MoLV. However, SFFV(MoLV) was easily converted back to the LLV-F type after only one passage in BALB/c mice coinfected with NB-tropic LLV-F. The antigenic interconversion between LLV-F and MoLV types demonstrated that SFFV is defective with respect to the expression of neutralizable envelope antigens.

Analysis of the neutralizable envelope antigens of nine SFFV(MuLV) pseudotypes by a panel of seven typing antisera made possible a "type-specific" SFFV(MuLV) envelope classification. Two major categories have been identified which correspond to the Gross (G) and Friend-Moloney-Rauscher (FMR) subgroups. Further, the FMR subgroup was divided into four types on the basis of distinct neutralization patterns. These results indicated that the specificity observed by cytotoxic G vs. FMR antisera is different from that observed by neutralization kinetics. We therefore suggest that the specific antigens revealed by virus neutralization tests be referred to as type specific.

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