Quantitation of Friend Spleen Focus-Forming Virus by a Nine-Day ^{59}Fe Assay

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A previously described 3-day ^{59}Fe assay for quantitation of Friend spleen focus-forming virus has been modified to produce a 200-fold more sensitive 9-day ^{59}Fe assay. A characterization of this assay is reported here. Male BALB/c mice received intravenous injections of appropriately diluted Friend polycythemia virus (FVP); control mice received virus diluent. All mice were allowed food and water *ad libitum* for 6 days, and on day 6 after virus injection were fasted by removal of food but not water. On day 3 of the fast (the 9th day after virus injection) each mouse received an intraperitoneal injection of 1 μCi of ^{59}Fe . Six hours later the mice were sacrificed and the splenic radioactivity was determined. The percent splenic incorporation of ^{59}Fe was directly related to the logarithm of spleen focus-forming units (SFFU) of FVP injected in a range of approximately 25 to 1,000 SFFU. Using a standard FVP preparation in a dose range of 25 to 1,000 SFFU, it was possible to determine the SFFU titers of unknown samples by extrapolation of the percent splenic ^{59}Fe incorporation to the logarithm of SFFU. SFFU titers obtained by the 9-day ^{59}Fe assay were similar to those obtained by the enumerative-response assay. Advantages of the 9-day ^{59}Fe assay over the enumerative-response assay include a 50-fold greater virus dose range, an easier and a more objective counting procedure, and a reduced coefficient of variation.

Induction of polycythemia in mice by the polycythemia strain of Friend virus (FVP) is the result of the combined effects of the spleen focus-forming virus (SFFV) considered defective (3-6) and its "helper," lymphocytic leukemia virus. Collectively these viruses comprise the Friend virus complex. Evidence is available that indicates that the defective SFFV is responsible for the transformation of erythroid cells, whereas the helper lymphocytic leukemia virus is believed to be necessary for providing the viral envelope of the SFFV required for infectivity (4, 6). Quantitative assays for lymphocytic leukemia virus include the XC-assay of Klement et al. (12), as well as other methods such as the technique of Bassin et al. (2). Assays for SFFV are based on a splenic erythroid response to the Friend virus complex. Such assays include the enumerative-response assay, referred to as the SFFV assay, described by Axelrad and Steeves (1), and a 3-day ^{59}Fe assay, described by Hankins and Krantz (9).

The enumerative-response assay is based on the induction of macroscopic foci on the surface of the intact mouse spleen. In this procedure,

mice receive intravenous (i.v.) injections of appropriately diluted virus. After 9 days the mice are sacrificed and their spleens are excised. The spleens are fixed in Bouin solution, and the foci on the surface of the spleens are counted. SFFV titers are calculated on the basis of the focal counts and are expressed as spleen focus-forming units (SFFU) per milliliter.

The 3-day ^{59}Fe assay is based on the capacity of SFFV to increase the splenic uptake of ^{59}Fe . In this procedure mice are injected i.v. with dilutions of FVP and are then fasted for 3 days. Fasting has been shown to reduce the endogenous level of erythropoietin-dependent erythropoiesis (7, 16), thus facilitating the measurement of virus-dependent erythropoiesis which is independent of erythropoietin (9, 13). On day 3 after virus injection, each mouse receives an intraperitoneal (i.p.) injection of ^{59}Fe . After 6 h the spleens are excised and the incorporation of ^{59}Fe is determined by counting in a well-type gamma scintillation counter. From the relationship between a standard amount of SFFU injected and the splenic uptake of ^{59}Fe , unknown titers are determined. Although the 3-day ^{59}Fe assay is much more rapid than the enumerative-response assay, it is far less sensitive.

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We have found that the sensitivity of the ^{59}Fe assay can be greatly enhanced by adopting the 9-day period of the enumerative-response assay and fasting for the last 3 days of this period. A characterization of this assay is the subject of this report.

MATERIALS AND METHODS

Mice. Male BALB/c mice between 6 and 8 weeks of age (Cumberland View Farms, Clinton, Tenn.) were used for quantitation of Friend SFFV.

Virus. FVP was obtained from R. Holdenried, National Institutes of Health, Bethesda, Md. Stock FVP consisted of infectious BALB/c mouse plasma prepared as previously described (10). Stock virus was stored in 0.4-ml portions at -80°C until used.

Radioisotopes and chemicals. $^{59}\text{FeCl}_3$ (15 to 30 mCi/mg) was obtained from ICN Life Sciences, Irvine, Calif. $^{59}\text{FeCl}_3$ was diluted in Hanks balanced salt solution to achieve $5 \mu\text{Ci/ml}$. Carboxymethylcellulose (CM-52, microgranular and preswollen) was obtained from Reeve Angel and Co., Clifton, N.J. (manufactured by Whatman, Ltd., England).

Assays for SFFV: enumerative-response assay. The SFFV titer of the standard FVP plasma preparation used in the 9-day ^{59}Fe assay was determined according to the method of Axelrad and Steeves (1) using 8 to 10 mice per virus dilution. Virus titers are expressed as SFFU per milliliter.

Nine-day ^{59}Fe assay. The 9-day ^{59}Fe assay for SFFV is based on a previously described 3-day method (9). Using Hanks balanced salt solution as virus diluent, five to eight mice were injected i.v. with 0.2 ml of each dose (25, 50, 500, and 1,000 SFFU) of standard FVP plasma using the lateral tail vein. A similar number of mice received an i.v. injection of 0.2 ml of virus diluent and served as controls. Samples of putative virus were appropriately diluted and 0.2 ml was injected i.v. into groups of five to eight mice. All mice were allowed food and water ad libitum for 6 days and then were fasted by removal of food but not water from days 6 to 9. On the 9th day after virus injection (3rd day of the fast) each mouse received an i.p. injection of $1 \mu\text{Ci}$ of ^{59}Fe in a volume of 0.2 ml. After 6 h, the spleens were excised and placed into small tubes containing 2 ml of phosphate-buffered saline. The splenic radioactivity was determined by counting in a well-type gamma scintillation counter (Nuclear Chicago Corp., Des Plaines, Ill.). The mean percent splenic ^{59}Fe was then plotted as a function of the logarithm of SFFU of the standard FVP plasma injected. Using the standard curve, SFFU titers of unknown samples were determined by extrapolation of the mean percent splenic ^{59}Fe incorporation to the logarithm of SFFU. SFFU titers represent the mean of two doses of injected material.

CMC chromatography. The amount of ^{59}Fe -labeled hemoglobin in fasted FVP-injected and fasted control mice was determined using a modification of the carboxymethylcellulose (CMC) microchromatographic procedure of Schroeder et al. (19). Four mice were injected with FVP and four control mice were injected with virus diluent. Mice were fasted from

days 6 to 9 after virus injection and then were injected i.p. with $1 \mu\text{Ci}$ of ^{59}Fe on day 9. After 6 h the spleens were excised. Each spleen was then homogenized in 2 ml of Drabkin solution diluted 1:2 with buffer at pH 6.1 [0.03 M tris(hydroxymethyl)amino-methane (Tris)-hydrochloride, 0.025 M NaCl, 0.01% KCN] and the homogenate was then clarified by centrifugation at $1,500 \times g$ for 5 min. Supernatant fluids were collected, and after the total radioactivity was determined 0.2 ml of each spleen homogenate was chromatographed. Using the same buffer at pH 6.1 and collecting 1-ml fractions, the nonhemoglobin ^{59}Fe was eluted, usually in fractions 1 through 7. Buffer at pH 7.5 (0.03 M Tris-hydrochloride, 0.04 M NaCl, 0.01% KCN) was then added and the hemoglobin was eluted, usually in fractions 9 through 11. The absorbance at 415 nm was measured and the radioactivity was determined in a well-type gamma scintillation counter.

Density gradient centrifugation and fractionation. A linear gradient of sucrose (Fisher Scientific Co.) from 15 to 60% (wt/vol) (0.01 M Tris-hydrochloride, pH 7.6; 0.14 M NaCl; 0.001 M KCl; 0.005 M MgCl_2) was prepared to a total volume of 4.7 ml. To this gradient 0.5 ml of FVP plasma was applied, and the gradient was centrifuged for 18 h at $180,000 \times g$ at 4°C in a Beckman SW50.1 rotor using a Beckman L2-50 ultracentrifuge. This gradient was then fractionated with an ISCO sucrose gradient fractionator by displacement with 60% (wt/vol) sucrose, and 0.25-ml fractions were collected from the top. Each fraction was diluted 10-fold in Hanks balanced salt solution, and eight mice were injected i.v. with 0.2 ml of each diluted fraction. A 9-day ^{59}Fe assay was then carried out.

RESULTS

Relation of splenic ^{59}Fe incorporation and SFFU. Increasing concentrations of a standard FVP plasma were injected i.v. into groups of eight BALB/c mice. Control mice received i.v. injections of virus diluent. A 9-day ^{59}Fe assay was then performed. As shown in Fig. 1, the mean percent splenic ^{59}Fe incorporation was virus dose-dependent and was directly related to the logarithm of the SFFU injected in a dose range of approximately 25 to 1,000 SFFU. The validity of using the SFFU as the standard unit and correlating the logarithm of the SFFU injected with the percent splenic ^{59}Fe incorporation is shown in Fig. 2. Three curves were obtained when the mean percent splenic ^{59}Fe incorporation induced by increasing doses of three FVP plasmas was plotted as a function of the volume of plasma injected into replicate groups of mice (Fig. 2A). However, as can be seen in Fig. 2B, a single curve equating splenic ^{59}Fe uptake and virus dose was obtained when these preparations were assayed for spleen focus-forming activity by the enumerative-response assay, and the mean percent splenic ^{59}Fe incorporation was plotted as a function of the logarithm of SFFU injected.

CMC chromatography of splenic homogenates. Although a direct relation was established between the splenic uptake of ^{59}Fe and the logarithm of the SFFU injected in a finite dose range, additional evidence was sought to determine if this relation was based on a splenic erythroid response to the virus. BALB/c mice were injected i.v. with FVP or virus diluent and a 9-day ^{59}Fe assay was performed. Clarified spleen homogenates from the infected and control mice were then subjected to CMC chromatography and the percentage of radioac-

FIG. 1. Relation of splenic ^{59}Fe incorporation and SFFU. Groups of eight male BALB/c mice were injected with increasing doses of the standard FVP plasma and a 9-day ^{59}Fe assay was performed. The logarithm of the SFFU injected was then plotted as a function of the mean percent splenic $^{59}\text{Fe} \pm$ the standard error of the mean (S.E.M.). Also shown is the virus dose range of the enumerative-response assay as a comparison to that of the 9-day ^{59}Fe assay.

tive hemoglobin was determined (Fig. 3). Figure 3A indicates that ^{59}Fe -labeled hemoglobin (eluted in fractions 9 through 11) was not detected in the control spleen homogenates. In contrast, the spleen homogenates prepared from the infected mice contained approximately 9 to 10% ^{59}Fe -labeled hemoglobin. When compared to the control mice, the spleen homogenates from FVP-infected mice also contained an increased amount of nonhemoglobin ^{59}Fe . It is possible that some of the nonhemoglobin ^{59}Fe was also in erythroid cells since other molecular species containing ^{59}Fe have been associated with erythrocyte formation (11). However, the identity of the nonhemoglobin ^{59}Fe species in the FVP-infected spleens is unknown. The absorbance profiles at 415 nm of the CMC-chromatographed spleen homogenates are shown in Fig. 3B. The absorbance profile is similar to the ^{59}Fe elution profile. Spleen homogenates prepared from FVP-infected mice had an increased absorbance at fraction 10, whereas little absorbance was observed with fractions 9 and 10 of the control spleen homogenates.

Comparison of the 9-day ^{59}Fe assay to the enumerative-response assay. The mean virus titers of unknown plasmas were determined by both the 9-day ^{59}Fe assay and the enumerative-response assay. Table 1 shows a comparison of the SFFU titers obtained with three FVP plasmas when assayed by the 9-day ^{59}Fe assay and by the enumerative-response assay. As can be seen, the variation in SFFU titers was two-fold or less.

Both the 9-day ^{59}Fe and the enumerative-response assay were analyzed for inherent variability by measuring the coefficient of variation (18). The analyses were performed on five FVP plasmas using three or four virus doses within

FIG. 2. Use of SFFU as the standard unit. (A) Groups of seven male BALB/c mice were injected with increasing volumes of three different FVP plasmas. A 9-day ^{59}Fe assay was then carried out and the mean percent splenic ^{59}Fe was plotted as a function of the volume of plasma injected. (B) The SFFU titer of each plasma was then determined using the enumerative-response assay and the percent splenic ^{59}Fe was plotted as a function of the logarithm of SFFU. The standard errors of the mean were similar to those in Fig. 1 and were omitted for clarity.

FIG. 3. CMC chromatography of spleen homogenates prepared from FVP-infected and uninfected control mice. Two-tenths milliliter of each clarified spleen homogenate was applied to two CMC columns, eluted with buffer at pH 6.1 (0.03 M Tris-hydrochloride, 0.025 M NaCl, 0.01% KCN) and 1-ml fractions were collected (fractions 1 through 7). Buffer at pH 7.5 (0.03 M Tris-hydrochloride, 0.04 M NaCl, 0.01% KCN) was then added to the columns and 1-ml fractions were collected (fraction 8 through 12). The radioactivity and absorbance at 415 were then determined for each fraction (A and B, respectively).

TABLE 1. Spleen focus-forming virus titers^a

FVP plasma	Method of assay	
	9-day ^{59}Fe assay	Enumerative assay
A	4.2×10^5	5.4×10^5
B	2.4×10^5	1.19×10^5
C	2.38×10^5	2.30×10^5

^a Virus titers are expressed as spleen focus-forming units per milliliter.

the dose range of each assay. Values which were excluded from computations included spleens that had greater than 30 foci (enumerative-response assay) and spleens from virus-injected mice that had incorporated less ^{59}Fe than the control animals (9-day ^{59}Fe assay). The mean coefficient of variation for the 9-day ^{59}Fe assay and the enumerative-response assay was 0.333 and 0.556, respectively. The median coefficient of variation for the 9-day ^{59}Fe assay and the enumerative assay was 0.292 and 0.680, respectively. Nonparametric analysis (20) of these values indicated that they were significantly different ($P = 0.002$).

Utility of the 9-day ^{59}Fe assay. Figure 4 represents an experiment in which FVP plasma was sedimented by density gradient equilibrium centrifugation in a 15 to 60% linear sucrose gradient. After centrifugation the gradient fractions were collected, diluted, and as-

FIG. 4. Density gradient analysis of standard FVP plasma. Five-tenths milliliter of the standard FVP plasma was layered on top of 4.7 ml of a 15 to 60% (wt/vol) linear sucrose gradient. The material was then centrifuged in an SW50.1 rotor at $180,000 \times g$ at 4°C for 18 h. Fractions were collected and diluted 10-fold in Hanks balanced salt solution. Eight male BALB/c mice were injected i.v. with 0.2 ml of each diluted fraction, and a 9-day ^{59}Fe assay was performed. Densities were determined by refractometry.

sayed for SFFV using the 9-day ^{59}Fe assay. The peak of virus infectivity was observed between 37.5 and 42.5% sucrose, corresponding to an approximate density range of 1.14 to 1.16 g/cm³.

DISCUSSION

The dose-response curve of the 9-day assay shows that as little as 25 SFFU significantly increased the splenic uptake of ^{59}Fe , indicating that this is a very sensitive method. In addition, the splenic uptake was directly related to the logarithm of SFFU injected in a range of 25 to 1,000 SFFU, providing a linear dose-response curve very suitable for an assay procedure. It is unlikely that the increased splenic uptake of ^{59}Fe observed in mice injected with FVP plasma was due to the presence of erythropoietin in the plasma. Several investigators have shown that antibody to erythropoietin did not inhibit the effect of FVP plasma (9, 13). When FVP plasma was sedimented by equilibrium density centrifugation through a 15 to 60% linear sucrose gradient, the most potent fractions were in a density range of 1.14 to 1.16 g/cm³. This is a density range consistent with a type C virus particle (14), but inconsistent with the density of erythropoietin. Erythropoietin has a sedimentation constant of 4.6 (8) and would therefore be recovered from the top portion of the gradient where no activity was observed.

When SFFU titers for several FVP plasmas were determined by both the enumerative-response assay and the 9-day ^{59}Fe assay, a twofold or less difference was observed. This degree of difference is considered the acceptable level for the enumerative-response assay (1). Since the two assays appear to yield similar virus titers they are quite comparable; however, the 9-day ^{59}Fe assay offers several advantages over the enumerative-response assay and the 3-day ^{59}Fe assay.

The 9-day ^{59}Fe assay is approximately 200-fold more sensitive than the 3-day ^{59}Fe assay and as such requires much less virus. This greatly reduces the total cost of mice required to produce stock FVP plasma, as well as reducing the quantity of unknown preparations to be assayed. With the extended virus dose range of the 9-day ^{59}Fe assay there is less chance that an assay will have to be repeated using more concentrated specimens. The major disadvantage of the 9-day ^{59}Fe assay compared with the 3-day ^{59}Fe assay is the increased time required.

The dose range of the 9-day ^{59}Fe assay is approximately 50-fold greater than that of the enumerative-response assay. In addition, the 9-day ^{59}Fe assay utilizes an easier and more objective counting procedure. The enumerative-response assay is based on the manual counting of splenic foci, a process which is subjective with respect to focus identification and also is time consuming and tedious. The 9-day ^{59}Fe assay, on the other hand, uses the automated

measurement of splenic radioactivity to determine SFFU. When titrating samples for SFFU the 9-day ^{59}Fe assay requires fewer mice. This is due in part to its extended virus dose range, which increases the likelihood of being in the countable range of virus activity, but also due to its reduced variability as indicated by a lower coefficient of variation. The major disadvantage of the 9-day ^{59}Fe assay compared with the enumerative response assay is its slightly decreased sensitivity.

The 9-day ^{59}Fe assay is a simple and precise method for quantitating Friend SFFV. Due to the wider virus dose range, objective counting procedure, and reduced coefficient of variation, we have found the 9-day ^{59}Fe assay more useful than the enumerative-response assay, especially when assaying a large number of samples.

Since the 9-day ^{59}Fe assay for Friend SFFV is based on splenic erythroid response to SFFV (1), this assay probably also could be used to quantitate Rauscher leukemia virus (Rauscher SFFV), a virus which also induces a splenic erythroid response (17) and is frequently quantitated by its capacity to induce splenic foci (15).

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