Production of Spleen Focus-forming Virus and Murine Leukemia Virus by Early Erythroblasts after Friend Virus Infection

Thomas A. Kost, W. David Hankins, A. D. Glick, and Sanford B. Krantz

Departments of Medicine [T. A. K., W. D. H., S. B. K.] and Pathology [A. D. G.], Vanderbilt University School of Medicine and Veterans Administration Hospital [S. B. K.], Nashville, Tennessee 37232

ABSTRACT

Spleen cells removed from BALB/c mice as soon as 48 hr after infection with Friend polycythemia virus were separated by velocity sedimentation at unit gravity. Cell fractions were assayed for the production of spleen focus-forming virus and for Friend murine leukemia virus. The production of both viruses per nucleated cell was up to 100-fold greater by the fractions containing cells of highest sedimentation velocity and of which 80 to 90% were early erythroblasts, as compared to the fractions with cells of low sedimentation velocity of which fewer than 20% were erythroblasts. When spleen cells were obtained from mice at 48, 72, or 120 hr after Friend polycythemia virus infection, similar patterns of virus production were observed. Electron microscopy revealed C-type virus particles budding from the surface of the erythroblasts. Increased virus production by the rapidly sedimenting cells enriched in early erythroblasts was not due to the removal of inhibitory cells since inhibition of virus production was not observed after mixing the fractions enriched for early erythroblasts with cells from other areas of the gradient that were deficient in virus production. When the cells in the peak-virus-producing fractions were incubated in vitro in suspension culture, they produced a wave of hemoglobin synthesis, the time course of which closely approximated the rate of virus production. Cells from the same fractions also formed erythroid colonies without added erythropoietin in plasma clot cultures. These studies indicate that, as early as 48 hr after Friend polycythemia virus infection, the greater part of both spleen focus-forming virus and Friend murine leukemia virus found in the spleen is produced by early erythroblasts.

INTRODUCTION

The infection of genetically susceptible mice with FV results in a rapid increase in erythropoiesis (20, 25). Friend virus preparations are known to contain at least 2 biologically active viruses. The virus that produces erythroid cell transformation is referred to as SFFV because of its capacity to induce discrete macroscopic foci of erythroid cells in the red pulp of the spleen (1). The production of infectious SFFV by infected cells requires the presence of MuLV-F (6, 8, 26). The infection of susceptible mice with MuLV-F in the absence of SFFV leads to the onset of a leukemic state, but the leukemia develops slowly, and no marked increase in erythropoiesis is evident (6, 9).

Recent studies in our laboratory (13) have shown that spleen cells obtained from plethoric mice soon after FV infection significantly increase their rate of hemoglobin synthesis when cultured in vitro. Spleen cells from uninfected mice show no such increase. The increase in the rate of hemoglobin synthesis consists of a well-defined wave that occurs 3 to 5 days postinfection, irrespective of the dose of virus injected or the time at which the FV cultures were prepared subsequent to infection (14). Further experiments have shown that the observed wave of hemoglobin synthesis is accompanied by a parallel increase in the production of infectious SFFV and MuLV-F (18). The increase and subsequent decline of both hemoglobin synthesis and virus production do not appear to be due to culture conditions but rather to an inherent characteristic of the infected spleen cells in this culture system. Studies by other investigators using electron microscopy have shown that, after FV infection, erythroblasts and reticulocytes had C-type particles budding from their cytoplasmic membranes (16, 28). Kuzumaki et al. (15) detected C-type particles budding from the cell membrane of lymphoid and erythroid precursor cells obtained from newborn rats that had been infected with MuLV-F. Feldman (10) reported that, following EPO stimulation of mice, increased C-type particle formation was found associated with erythroblasts. However, in these studies, the functional identity of the virus associated with the erythroid cells was not determined. Our findings, that new erythropoiesis occurred concomitantly with SFFV and MuLV-F production, suggested that both SFFV and MuLV-F were being produced by new erythropoietic cells that resulted from FV transformation (18). We now report further studies in which the early splenic erythroblasts from FV-infected mice were enriched by velocity sedimentation at unit gravity and were assayed for both MuLV-F and SFFV production. These experiments indicate that the early erythroblasts found in the spleen as soon as 48 hr after infection are the splenic cells most actively involved in the production of both viruses.

MATERIALS AND METHODS

Mice. Male BALB/c mice, between 8 and 10 weeks old were obtained from the National Cancer Institute, Frederick, Md.

Virus. Stock FV plasma consisted of infectious BALB/c mouse plasma prepared and stored as described previously (12). MuLV-F was assayed by the XC syncytial plaque assay utilizing duplicate plates, and titers were expressed in terms of PFU (23). SC-1 cells were obtained from the Naval Bioscience Laboratory, Oakland, Calif., and XC cells were obtained from...
The spleen focus-forming assay was used to quantitate SFFV activity in terms of FFU (1). Groups of 5 to 7 mice were used for all SFFV assays. The FV plasma contained $4.0 \times 10^7$ PFU/ml and $3.1 \times 10^8$ FFU/ml of MuLV-F and SFFV, respectively.

**Preparation of FV-infected Spleen Cells.** Groups of 5 BALB/c mice were infected i.v. through the lateral tail vein with 100 µl of FV plasma diluted in HBSS to yield an injection volume of 0.4 ml. At the designated time after virus infection, the mice were sacrificed, and the spleens were excised and rinsed in cold HBSS. The spleen capsules were scored at several points, placed into nylon bags cut from platelet recipient sets Hb-182 (Fenwal Inc., Ashland, Mass.) and submersed in HBSS. The spleen cells were expelled using a blunt object, collected in HBSS, and pelleted by centrifugation at 1000 x g for 5 min at 4°. The pellet was suspended in HBSS, washed once, and resuspended in 0.2% PBS prior to cell separation. The BSA was deionized by the method of Worton et al. (27).

**Cell Separation.** Spleen cells were separated by velocity sedimentation at unit gravity with a method similar to that described by Miller and Phillips (19). The sedimentation chamber (O. H. Johns Glass, Toronto, Ontario, Canada) was cylindrical with a diameter of 18 cm and with a depth of 7 cm. The chamber had a conical base with a 2.5-mm opening at the base of the cone. Turbulence occurring upon filling or emptying of the chamber was minimized by a stainless steel flow deflector. All operations were carried out at 4°. The chamber was loaded with 40 ml of PBS followed by 40 ml of a cell suspension containing 4 to 6 x 10^8 cells in PBS with 0.2% BSA. A linear 1 to 2% BSA gradient in a total volume of 1240 ml was then allowed to flow into the chamber. The cells were allowed to sediment for 3.5 hr prior to draining the chamber. The first 350 ml that occupied the conical part of the chamber was discarded, and the remainder was collected in twenty-four 40-ml fractions. Nucleated cell counts were performed in 0.1% methylene blue with a hemocytometer. The cells in each fraction were pelleted by centrifugation at 1000 x g for 5 min. The cell pellet was suspended in MEM containing 20% fetal bovine serum. A portion of the cells were frozen immediately at —70°. The remaining cells were placed in 60- x 15-mm Petri dishes and incubated at 37° in 5% CO2 in a humidified atmosphere for the indicated time periods. After incubation, the cell suspensions were collected and were frozen immediately at —70°. Prior to assaying for SFFV and for MuLV-F, the suspensions were thawed at 37° and filtered through 0.45-µm filters.

**Cell Morphology.** Slides were prepared with a cytocentrifuge (Shandon Scientific Co., London, England) and stained with Wright’s stain or with benzidine-Wright-Giemsa stain (24). For electron microscopy, the spleen cells were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded alcohol solutions, and transferred through propylene oxide to Araldite. The sections were stained with uranyl acetate and medium were collected, and the content of infectious SFFV and MuLV-F was measured. A typical nucleated cell profile is shown that the rate of heme synthesis determined by this procedure represents the rate of hemoglobin synthesis (12, 14).

**RESULTS**

**SFFV and MuLV-F Production by Spleen Cells after Velocity Sedimentation at Unit Gravity.** BALB/c mice each received 0.1 ml of FV plasma, and after 48, 72, and 120 hr the mice were sacrificed and spleen cell suspensions were prepared. The cells were layered over 1 to 2% gradients of BSA and were incubated at 37° for 5 hr before fractions were collected and nucleated cell counts were made. The remaining cells in each fraction were suspended in culture medium and incubated for 6 hr at 37°. At this time, the cells and medium were collected, and the content of infectious SFFV and MuLV-F was measured. A typical nucleated cell profile is shown in Chart 1A. The majority of nucleated cells had a modal sedimentation velocity of 3.44 mm/hr with less than 10% of the cells sedimenting below Fraction 10. The uppermost fractions (Fractions 21 to 24) contained mainly erythrocytes, dead cells, and debris. Viability of the nucleated cells below Fraction 20 was between 85 and 90% as determined by trypan blue exclusion. As evident from Chart 1B and C, the production of SFFV and MuLV-F on a per cell basis was highest by the cells that sedimented below Fraction 10. The uppermost fractions (Fractions 21 to 24) contained mainly erythrocytes, dead cells, and debris. Viability of the nucleated cells below Fraction 20 was between 85 and 90% as determined by trypan blue exclusion. As evident from Chart 1B and C, the production of SFFV and MuLV-F on a per cell basis was highest by the cells that sedimented below Fraction 10. The uppermost fractions (Fractions 21 to 24) contained mainly erythrocytes, dead cells, and debris. Viability of the nucleated cells below Fraction 20 was between 85 and 90% as determined by trypan blue exclusion. As evident from Chart 1B and C, the production of SFFV and MuLV-F on a per cell basis was highest by the cells that sedimented below Fraction 10. The uppermost fractions (Fractions 21 to 24) contained mainly erythrocytes, dead cells, and debris. Viability of the nucleated cells below Fraction 20 was between 85 and 90% as determined by trypan blue exclusion. As evident from Chart 1B and C, the production of SFFV and MuLV-F on a per cell basis was highest by the cells that sedimented below Fraction 10. The uppermost fractions (Fractions 21 to 24) contained mainly erythrocytes, dead cells, and debris. Viability of the nucleated cells below Fraction 20 was between 85 and 90% as determined by trypan blue exclusion. As evident from Chart 1B and C, the production of SFFV and MuLV-F on a per cell basis was highest by the cells that sedimented below Fraction 10.

**Culture of Erythroid Colonies.** Spleen cells from gradient fractions were cultured at a concentration of 1 x 10^5 nucleated cells/ml in MEM alpha medium (with ribosides and deoxyribose) containing 30% heat-inactivated fetal bovine serum, 1% deionized BSA, 0.85% bovine embryo extract, L-asparagine (0.02 mg/ml), 10^{-4} M β-mercaptoethanol, penicillin (100 IU/ml), streptomycin (100 µg/ml), and 10% bovine citrated plasma. The cells were dispersed in 0.1-ml aliquots (in quadruplicate) into the wells of microtiter plates (No. 1-220-24; Cooke Laboratory Products, Alexandria, Va.) and were incubated at 37° in a humidified atmosphere containing 5% CO2 in air (17). After 48 hr of incubation, the clots were placed on glass slides and fixed with 5% glutaraldehyde in 0.01 M phosphate buffer (pH 7.0 to 7.2). The slides were stained in a 1% solution of 3,3'-dimethoxybenzidine before being placed in 2.5% hydrogen peroxide in ethanol. The slides were counterstained with Harris' hematoxylin and washed in tap water (17). Colonies of 8 or more benzidine-positive cells were scored at x100.

**Preparation of 56Fe Solution and Extraction of Heme.** Iron-free plasma was prepared as described previously (14). The plasma was combined with MEM (40 and 60%, respectively) and 56FeCl3 (specific activity, 15 to 30 µCi/mg) was added to achieve 10 µCi/ml. To measure 56Fe incorporation in heme, 12 µl of this solution were added to each of 4 wells of a tissue culture multiwell plate (No. 76-002-95; Linbro Scientific, Hamden, Conn.) containing 1 x 10^8 spleen cells in MEM with 20% fetal bovine serum. The cells were collected, and 56Fe incorporation into heme was determined after extraction into cyclohexanone as described previously (14). Previous work has shown that the rate of heme synthesis determined by this procedure represents the rate of hemoglobin synthesis (12, 14).
ing times of infection; therefore, it is likely that a higher number of virus-producing cells was present in these fractions.

**Morphology of Spleen Cells That Produce Large Amounts of Virus.** Spleen cells were removed from mice 72 hr after infection with FV and were fractionated as described in the preceding experiment. Following sedimentation, fractions were collected, and the cell types present in various areas of the gradient were determined. Slides were prepared from each pooled fraction using a cytocentrifuge and were stained by the conventional Wright’s stain method or with benzidine-Wright-Giemsa. Cells were also examined by electron microscopy. SFFV and MuLV-F were measured after 6 hr incubation at 37°. The predominant cells in the lower fractions of the albumin gradient (below Fraction 10) were large immature cells with a deep basophilic cytoplasm, prominent nucleoli, and chromatin structure typical of early erythroblasts (Fig. 1A). These cells comprised 80 to 90% of the total cells in the lower fractions (Chart 2B). The remaining 10 to 20% of the cells consisted primarily of polychromatophilic and orthochromatophilic erythroblasts, lymphocytes, and granulocytes. The identification of the majority of the cells in the lower fractions as early erythroblasts was confirmed by the electron microscopic appearance of the cells (Fig. 1B). The cells had prominent nucleoli, numerous ribosomes in the cytoplasm, and phagocytoxic vesicles at the cell periphery. C-type virus particles were observed budding from the cell surface of most of the erythroblasts (Fig. 1C). As described in the preceding experiment, production of both SFFV and MuLV-F was greatest by the rapidly sedimenting cells in the lower fractions (Chart 2A). The nucleated cells that sedimented between Fractions 10 and 20 consisted mainly of late orthochromatophilic erythroblasts and lymphocytes, with less mature erythroblasts comprising less than 10% of the erythroblasts present.

**Kinetics of SFFV and MuLV-F Production by Separated Spleen Cells.** In the preceding experiments, virus production by the separated spleen cells was determined by incubating all of the cells of a particular fraction for 6 hr at 37°. This resulted in the cells throughout the gradient being cultured at a variety of concentrations ranging from 1 x 10^6 cells/ml at the bottom of the gradient to 6 x 10^6 cells/ml at the top, since fewer cells sedimented to the bottom of the gradient. To eliminate the possibility that the rapidly sedimenting cells were producing more virus on a per cell basis because of their lower cell concentration as compared to that of the more slowly sedimenting cells, spleen cells from 72-hr FV-infected mice were sedimented, and the fractions were adjusted for culture at a similar cell concentration of 1.7 x 10^6 cells/ml. The production of both viruses was measured after 6, 18, and 42 hr of incubation (Chart 3B). The incubation of the cells from all fractions at a similar concentration emphasized the increased production of both viruses by the rapidly sedimenting cells. The amount of SFFV and MuLV-F produced by the rapidly sedimenting cells on a per cell basis after 18 hr incubation was over 100-fold greater than that produced by the cells in the upper half of the gradient or that produced by unseparated spleen cells incubated at a similar cell concentration. The amounts of SFFV and MuLV-F produced by unseparated cells after 18 hr incubation were 6 FFU and 29 PFU, respectively, per 10^6 nucleated cells. This is in contrast to 7.7 x 10^6 FFU and to 2.6 x 10^4 PFU per 10^6 cells produced by the cells in Fraction 8 after 18 hr of incubation. Virus production increased between 6 and 18 hr of incubation and declined by 42 hr of incubation. The observed time course of virus production is similar to that reported previously for spleen cells obtained from plethoric mice in...
Effect of Mixing Spleen Cell Fractions Obtained by Velocity Sedimentation on Virus Production. The reduced level of SFFV and MuLV-F production by the slowly sedimenting spleen cells could be due to the presence of an inhibitory cell or to the cellular product present in these fractions of the albumin gradient. To examine this possibility, spleen cells were obtained from mice 74 hr after infection with FV and were separated as in the prior experiments. Fractions 1 to 6 were pooled as were Fractions 15 to 20. These fractions represent cells which produced large and small quantities of virus, respectively, after 18 hr in vitro. As seen in Table 1, incubating cells from the pooled Fractions 1 to 6 at increasing concentrations resulted in a corresponding increase in the amount of infectious virus produced. Cells from the pooled Fractions 15 to 20 produced only low levels of virus but also showed increased virus production when incubated at increased cell concentrations. The incubation of increasing concentrations of cells of the pooled Fractions 15 to 20 with a constant amount of cells of the pooled Fractions 1 to 6 did not result in a reduced level of virus production.

Heme Synthesis and Erythroid Colony Formation by Separated Spleen Cells. The spleen cell fractions were examined for their capacity to synthesize hemoglobin and to form erythroid colonies in the absence of added EPO. Spleen cells were removed from 74-hr FV-infected mice and were sedimented as described previously. After 3.5 hr, fractions were collected and pooled; the cells from the pooled fractions were incubated at 37°C at a concentration of 1 x 10^6 cells/ml, and the incorporation of 59Fe into heme was measured for 6-hr periods during the incubation (Chart 4). The cells identified as 80 to 90% early erythroblasts that sedimented to the lower portion of the gradient (Fractions 1 to 8) initially incorporated only low levels of 59Fe into heme. However, incorporation began to increase rapidly and reached a maximum at 107 hr after infection. Thereafter, the rate of heme synthesis declined. The cells that sedimented between Fractions 9 and 16 consisting of fewer erythroblasts and a smaller percentage of virus-producing cells did not show an increase in heme synthesis. Initial levels were in the range of the cells in Fractions 1 to 8, but throughout the incubation period heme synthesis decreased steadily. A similar profile was observed for the unseparated spleen cells. The cells in Fractions 17 to 20 were found to have a high level of

<table>
<thead>
<tr>
<th>Rapidly sedimenting Fractions 1–6</th>
<th>Slowly sedimenting Fractions 15–20</th>
<th>Mixture (x 10^5 cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(x 10^4 cells/ml)</td>
<td>(x 10^4 cells/ml)</td>
<td>Observed titer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FFU (x 10^6)</td>
</tr>
<tr>
<td>1.5</td>
<td>1.0</td>
<td>3.1</td>
</tr>
<tr>
<td>3.0</td>
<td>3.5</td>
<td>5.8</td>
</tr>
<tr>
<td>4.5</td>
<td>5.1</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>0.046</td>
</tr>
<tr>
<td>15</td>
<td>0.15</td>
<td>0.079</td>
</tr>
<tr>
<td>30</td>
<td>0.23</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>2.1</td>
<td>2.9</td>
</tr>
<tr>
<td>1.5</td>
<td>2.4</td>
<td>3.2</td>
</tr>
<tr>
<td>1.5</td>
<td>2.8</td>
<td>3.5</td>
</tr>
<tr>
<td>1.5</td>
<td>3.1</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Determined by spleen focus-forming assay; values are in FFU/10^6 nucleated cells.

* Determined by XC plaque assay; values are in PFU/10^5 nucleated cells.
heme synthesis initially, but the rate declined very rapidly throughout the incubation period. The initial high level of heme synthesis is most likely to be due to the large number of mature polychromatophilic and orthochromatophilic normoblasts in these fractions. An examination of the fractions for cells yielding erythroid colonies in the plasma clot system showed that the cells giving rise to erythroid colonies were found also in the lower fractions, i.e., below Fraction 10 (Chart 5). The observed erythroid colonies were similar in appearance to those formed from the EPO-dependent colony-forming-unit erythroid, however, the colonies formed in the absence of added EPO and, in general, contained more than 8 benzidine-positive cells. In Fractions 3 to 6, approximately 1 of 20 cells gave rise to an erythroid colony. This was an enrichment of approximately 17-fold over the unseparated cells which yielded 1 erythroid colony. This was an enrichment of approximately 17-fold over the unseparated cells which yielded 1 erythroid colony. The observed erythroid colonies were a minor fraction of the rapidly sedimenting cells, and the precise morphological identity was not determined.

**DISCUSSION**

The present studies indicate that, as early as 48 hr after FV infection, the greater part of both SFFV and MuLV-F found in the spleen is produced by early erythroblasts. Velocity sedimentation of spleen cells from FV-infected mice resulted in the separation of cell fractions containing between 80 and 90% early erythroblasts. The production of both SFFV and MuLV-F on a per cell basis was greatest by those fractions highly enriched in early erythroblasts. Virus production by the cells in these fractions was over 100-fold higher than virus production by cells in other areas of the sedimentation gradient or by the unseparated spleen cells. Electron microscopy revealed C-type virus particles budding mainly from the early erythroblasts in the rapidly sedimenting cell fractions. Fractions that contained few early erythroblasts but that contained a higher percentage of lymphocytes and mature erythroblasts produced only small quantities of virus. The lack of virus production by these cell fractions was not due to the presence of inhibitory cells in the fractions because mixing of these cells with the fractions enriched for early erythroblasts did not result in decreased virus production by the latter.

In addition to producing large quantities of infectious virus, the rapidly sedimenting cells, enriched for early erythroblasts, also manifested a wave of heme synthesis in vitro in the absence of added EPO. The time course of heme synthesis, with a peak at 110 hr after infection, was similar to that obtained previously with unseparated spleen cells from FV-infected mice (14, 18). Because of the enrichment of early erythroblasts in the lower portion of the gradient, we were able to observe the wave of heme synthesis by culturing far fewer cells than in earlier studies in which high cell numbers were cultured to observe the increase in heme synthesis (14, 18). The time course of SFFV and MuLV-F production, with peak production occurring between 90 and 124 hr after infection, approximated the time course of heme synthesis, which represents new hemoglobin production (12, 14). The pattern of virus production was also similar to that reported previously for unseparated spleen cells (18).

Culture of the rapidly sedimenting cells in the plasma clot system in the absence of added EPO resulted in the formation of erythroid colonies 2 days after initiation of the cultures. These data are in agreement with previous results which utilized unseparated spleen and bone marrow cells from FV-infected mice (16, 22).

The evidence presented indicates that the early erythroblasts which appear in the spleen as early as 48 hr after FV infection produce a large part of the SFFV and MuLV-F found in that organ. This is consistent with the recent experiments of Nassarallah and McGarry (21) who demonstrated increased plasma titers of FV concomitant with increased erythroid regeneration following busulfan treatment of mice. However, this does not preclude the possibility that SFFV and MuLV-F are produced by other cell types present in the spleen. FV has been shown to infect and to replicate in other cell types besides erythroid cells. The continuous replication of SFFV and MuLV-F in mouse embryo fibroblasts has been reported (2, 9). Clarke et al. (5) have described the isolation of an FV-producing nonerythroid cell line from the spleen of an FV-infected mouse. In addition, Cerny et al. (3, 4) have presented data which indicate that B-lymphocytes serve as sites of MuLV-F replication following infection; however, no experiments were performed to determine if these lymphocytes produce infectious SFFV.

The question as to whether the continuous synthesis of FV by the infected erythroblasts is necessary for the expression of hemoglobin synthesis and erythroid colony formation in the absence of added EPO is not answered by this study. It is possible that virus infection alone may trigger the sequence of events which causes an erythroid cell to differentiate. Erythroleukemic cell lines have been described that respond to dimethyl sulfoxide with a large increase in hemoglobin synthesis but without demonstrable SFFV production (7). SFFV-infected cell lines have also been described that produce SFFV but do not have measurable hemoglobin synthesis (5, 9). Thus, the occurrence of hemoglobin synthesis together with FV production may represent 2 separate effects of virus infection of a single cell.

**ACKNOWLEDGMENTS**

The authors thank Anne Hoos for providing excellent technical assistance.
REFERENCES


Fig. 1. Morphology of spleen cells producing large amounts of virus. Spleen cells were removed from mice 72 hr after infection with FV and sedimented as described in Chart 1. A, Wright-stained cytocentrifuge preparation of spleen cells from pooled Fractions 1 to 5 of gradient showing deep basophilic cytoplasm and prominent nucleoli. × 600. B, typical early erythroblast from pooled Fractions 1 to 5 with rhopheocytotic vesicle (arrow) and budding virus. × 13,300; C, higher magnification of early erythroblast showing rhopheocytotic vesicle (arrow) and C-type virus budding from the cell membrane. × 67,200.
Production of Spleen Focus-forming Virus and Murine Leukemia Virus by Early Erythroblasts after Friend Virus Infection

Thomas A. Kost, W. David Hankins, A. D. Glick, et al.