Expression of Friend Leukemia Virus and Spleen
Focus-forming Virus-specific Sequences in Erythroid Bursts and
Granulocyte-Macrophage Colonies from Spleen and Marrow of Mice
Infected with Friend Leukemia Virus

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ABSTRACT

A large number of studies have been carried out to identify the Friend leukemia virus (FV) target cell(s). In FV-infected mice, the kinetics of "primitive" erythroid burst-forming units (P-BFU-E) is perturbed, and their proliferative rate is enhanced. These results indirectly suggest, but do not prove, that cycling P-BFU-E may serve as FV target. In vitro infection studies showed that normal erythroid colony forming units (CFU-E) and "mature" erythroid burst-forming units (M-BFU-E) are targets for FV, while the largely out-of-cycle normal P-BFU-E are not. In an attempt to shed light on these aspects, we have evaluated the expression of viral cytoplasmic RNA sequences in pools of colonies generated by P-BFU-E and granulocyte-macrophage colony forming units (CFU-GM) from spleen and marrow of polycythemic Friend virus (FVP)-infected mice, as measured by liquid hybridization with FVP- or spleen focus-forming polycythemic virus (SFFV)-specific DNA probes. Moreover, similar assays were performed on RNAs derived from whole spleen or bone marrow from mice treated with FVP or the anemic strain of Friend virus (FVA). Control studies were performed on corresponding colonies and whole tissues from normal animals.

FVP- and SFFV-specific sequences are more abundant in RNA extracted from infected spleen as compared to marrow by a 10-fold factor. On the other hand, FVP and SFFV-specific sequences are expressed at a comparable level in both P-BFU-E- and CFU-GM-derived colonies from spleen or marrow of FVP-treated mice. Since in vitro spread of FVP infection was excluded by control studies with addition in culture of antibody to the viral glycoprotein with a molecular weight of 70,000 (gp70) these results indicate that P-BFU-E and CFU-GM are infected in vivo by FVP.

INTRODUCTION

At least 2 strains of FV are presently known. The first one (FVA) induces an erythroleukemia accompanied by splenomegaly and anemia (10), and the second one (FVP) induces the same type of erythroleukemia associated with polycythemia (20). Both strains consist of 2 components, a replication-defective, spleen focus-forming virus (SFFVp and SFFVap, respectively) and a helper murine leukemia virus (F-MuLVp and F-MuLVap, respectively) (32). The 2 components have been recently cloned (17, 21). Injection of F-MuLVp or F-MuLVap into newborn BALB/c or NIH/Swiss mice induces an erythroleukemia with splenomegaly and anemia (32). Treatment with SFFVap in association with any of different helper viruses induces in both newborn and adult susceptible animals a different erythroleukemia characterized by splenomegaly, erythroblasticosis, and polycythemia (18).

A large number of studies have been focused on the target cell(s) of FV. In vivo observations (30) initially suggested that the target may be represented by an early erythropoietic precursor. In this regard, in vitro clonogenic assays for hemopoietic stem and progenitor cells have been recently developed. These techniques allowed identification of early (BFU-E) and late (CFU-E) erythroid progenitors. The former, closely related to the myeloid stem cell (CFU-S), gives rise to large erythroid colonies ("bursts"), and the latter generates small clusters of erythroblasts (1). The BFU-E pool can be further subdivided into progenitors of either primitive or mature type (P- and M-BFU-E, respectively) (7). The erythropoietic process is thus of multistep nature and entails CFU-S → P-BFU-E → M-BFU-E → CFU-E erythroblast differentiation (23). In vitro cycling and differentiation of BFU-E is largely regulated by BPA (14, 33), while CFU-E kinetics is mainly controlled by EP (1). On the other hand, proliferation of granulocyte-macrophage progenitors (CFU-GM) is modulated by CSF (4). The erythroleukemia induced by FV is characterized by marked amplification of the splenic pool of P-BFU-E (26) and CFU-E (8, 22, 26), as well as by enhanced cycling of the former progenitors (26). CFU-E from mice treated with FVP or SFFVp pseudotypes proliferate in vitro without EP addition, while those from FV-treated animals proliferate in presence of exogenous EP (8, 22, 26). It has been recently shown that growth of P-BFU-E from FV-treated mice is at least in part independent of BPA addition (24). All these studies suggest, therefore, that P-BFU-E, colony-forming units, erythroid; CFU-S, colony-forming units, spleen; P-BFU-E, primitive burst-forming units, erythroid; M-BFU-E, mature burst-forming units, erythroid; BFU-E, burst-forming units, erythroid; BFU-E, primitive burst-forming units, erythroid; BP, burst-promoting activity; EP, erythropoietin; CFU-GM, colony-forming units, granulocyte-macrophage; CSF, colony-stimulating factors; GM, granulocyte-macrophage; cDNA, complementary DNA; gp70, viral glycoprotein with a molecular weight of 70,000.
BFU-E and CFU-E may serve as target cells for FV; the evidence, however, is only indirect and hence inconclusive.

It has been suggested that normal CFU-E and particularly M-BFU-E but not P-BFU-E are in vitro targets for FV (15); the in vitro observations, however, may not necessarily be extrapolated to the in vivo situation. Finally, the possibility that FV infects the myeloid stem cell and the GM lineage is fully untested thus far.

As a novel approach to investigate these uncertain aspects, we evaluated the expression of FV sequences in colonies generated by P-BFU-E and CFU-GM from marrow and spleen of FVP-infected mice. In particular, liquid hybridization assays were carried out with FVP- or SFFVp-specific DNA probes on cytoplasmic RNA extracted from pools of these colonies. Further assays were performed on RNA derived from whole marrow and spleen cells at different time intervals after FVP or FVA infection. Control studies were carried out on corresponding colonies and whole spleen or marrow from uninfected animals.

MATERIALS AND METHODS

**Viruses.** N-tropic cell-free homogenates of FVA and FVP, prepared as described (10), were passaged in our laboratory. Virus stock titers were, for FVP and FVA, $10^{-4.8}$ and $10^{-3.2}$ of the dose lethal to 50% of the population per 0.2 ml, respectively.

**Mice.** Female DBA/2 mice weighing 16 to 22 g (obtained from CNEN, Casaccia, Rome, Italy, or Charles River Italia, Caibo, Italy) were fed with laboratory pellets and tap water ad libitum. The animals received FVP or FVA i.p. (0.2 or 0.3 ml/mouse, respectively).

**Cloning of Hemopoietic Progenitors.** Erythroid bursts and GM colonies were grown in methylcellulose culture according to a slight modification of a previously reported method (25, 26, 28). The animals were killed by cervical dislocation under light after anesthetization. Nucleated cells were obtained from tibia (26) and spleen (13). Each 1-ml plate contained the following components in Iscove's modified Dulbecco's medium (Gibco-Biocult, Paisley, Scotland): methylcellulose (0.8% final concentration), α-thioglycerol (10⁻⁴ M), fetal calf serum (2%), penicillin (100 units/ml), and streptomycin (100 mg/ml). Nucleated cells were from tibia and spleen (13). Each 1-ml plate contained the following components in Iscove's modified Dulbecco's medium (Gibco-Biocult, Paisley, Scotland): methylcellulose (0.8% final concentration), α-thioglycerol (10⁻⁴ M), fetal calf serum (30%), and 1 x 10⁵ nucleated cells from marrow and spleen, respectively, and either EP (1.5 IU) or CSF (i.e., long-conditioned medium) (0.1 ml) (25). EP was either human urinary, semipurified as described previously (26) or sheep Step III (Connaught Medical Research Laboratories, Toronto, Ontario, Canada) containing 0.1 ng endotoxin per IU (25). These amounts of EP and CSF induced maximal growth of P-BFU-E and CFU-GM colonies, respectively. The plates were incubated in a humidified atmosphere of 5% CO₂ in air at 37°C. After 6 to 7 days of culture, all CFU-GM colonies containing a minimum of 50 to 100 cells were picked up by means of a fine Pasteur pipet and pooled together. In some studies, granulocyte and macrophage colony-forming units colonies were separately pooled. These clones contain, respectively, >90% cells of the granulocytic and macrophage monocytic series, as confirmed by control studies on colonies cytotoxic to and stained by standard procedures. Large erythroid bursts, derived from P-BFU-E and containing >200 cells, were collected and pooled after 8 days of culture. Colony identification was performed in situ (on the basis of standard morphological criteria, as confirmed by cytotoxic staining and staining of cells from pooled colonies). As generally reported and confirmed in our laboratory, M-BFU-E-derived bursts contain 50 to 200 cells, reach peak growth on Day 3 to 4 of culture, and are largely lysed by Day 7 to 8.

**Synthesis of FV cDNAs.** Total representative FV cDNA was prepared from FV released by F 4–6 cells (5), with the endogenous enzyme in a lysed virion incubation (9). The [³H]FVP cDNA was synthesized with calf thymus DNA hydrolysate as primer for an efficient transcription (31). FVP cDNA (1 ng/13,000 cpm) hybridizes up to 90% with 100-fold excess of 70S template RNA, as shown in Chart 1.

**Viral RNAs in Hemopoietic Lineages from FV-infected Mice**

**RESULTS**

**Preliminary Experiments.** Cytoplasmic RNA from FVP-producing cell line F 4–6 (5) was hybridized with FVP- or SFFVp-specific DNA probe (Chart 2). In line with results obtained with purified viral RNA (see "Materials and Methods"), the probes hybridized up to 90 and 75%, respectively, in the presence of an excess of cytoplasmic RNA. As expected, a smaller one-half CF₅₀ value is obtained with the total probe as compared to the SFFVp-specific one. Indeed, the former contains sequences recognizing both defective and helper virus whereas the latter recognizes sequences present only in the former component.
Studies on Whole Spleen and Bone Marrow from Infected or Normal Mice. In these experiments, we have comparatively evaluated the levels of FV expression in splenic and marrow cells at 20 days after FVP or FVA treatment.

In FVP-infected animals, splenic cells contain both FVP-(Chart 3, top) and SFFVp-specific (Chart 3, bottom) sequences at levels comparable with those in F 4-6 cells (Chart 2). However, the amount of viral RNA detected with the FVP-specific probe is 10- to 20-fold less in marrow than in spleen (Chart 3, top; Table 1). A comparable difference is observed when RNA is hybridized with the SFFVp-specific probe (Chart 3, bottom; Table 1).

A similar pattern has been observed for the expression of viral RNA in spleen and marrow after FVA treatment (Chart 4). However, the level of viral RNA in spleen (Chart 4, top) is 3- to 4-fold lower than in the FVP-infected organ (Chart 3, top) when evaluated with either probes (Table 1). Here again, expression of viral RNA sequences in marrow is less pronounced than in spleen, i.e., of borderline level as in control. It cannot be excluded, however, that FVP versus FVA differences are at least in part due to the different FV titer inoculated.

It should be emphasized that RNA extracted from FVP-infected spleens hybridizes up to 100% with FVP and SFFVp probe, while that from marrow hybridizes only up to 75% with the same probes (Chart 3). A similar difference may be observed for FVA-infected spleen and marrow (Chart 4), as well as for colonies derived from either tissues (see below).

FV expression in whole hemopoietic tissues at different times after infection is summarized in Table 1. RNA from spleens infected with FVP for 7 days contains virus-specific sequences at levels comparable to those detected 20 days after treatment. In contrast, RNA from spleens infected with FVA for 7 days shows a percentage of viral sequences 10-fold less as compared to 20 days splenic RNA. The different expression of the 2 strains early after infection indicates a more rapid progression of the FVP-induced disease.

It has been shown previously that sequences homologous to SFFVp viral RNA are present in normal mice susceptible to FV (2). Data in Table 1 confirm this observation, in that low levels of SFFVp- and FVP-specific RNA were observed in hemopoietic tissues from uninfected adult mice. Once more, splenic RNA apparently contains 3 times more viral-specific sequences as compared to marrow RNA.

Studies on Erythroid Bursts and GM Colonies from Infected or Normal Mice. Virus-specific RNA was measured in pooled erythroid bursts and GM colonies generated by P-BFU-E and CFU-GM from spleen and marrow at 20 days after FVP infection.

In order to prove that the assay of viral sequences in the colonies was not influenced by spread of FVP infection to uninfected noncontiguous cells in the culture dish, control experiments were carried out by in vitro addition of anti-gp70 serum. In this regard, anti-gp 70 serum (generously provided by Dr. Stuart Aaronson, National Cancer Institute, NIH, Bethesda, Md.) was added to methylcellulose plate (20 μl/dish) throughout the incubation period. This did not significantly modify the pattern of RNA hybridization against the FVP probe as compared to that obtained with RNA extracted from colonies grown in presence of normal serum, i.e., a typical experiment showed 0.0145 and 0.0140% of viral RNA in splenic colonies, respectively, incubated or not with anti-gp70 serum. In order to ensure that the amount of anti-gp70 used effectively neutralized FVP possibly released in culture, further control experiments were carried out. Thus, in vitro infection of normal hemopoietic cells by FVP and burst promotion without EP addition was obtained as described by Hankins et al. (11, 12). [The cell number was 4 × 10^6, and appropriate virus dilution was used, (~5000 SFFVp/ml.)] Addition of 20 μl of anti-gp70 serum in the FVP-plus-cells incubation step fully abolished burst formation upon cell plating.
**Table 1**

<table>
<thead>
<tr>
<th>Cytoplasmic RNA</th>
<th>% of hybridization</th>
<th>C_{t/2}</th>
<th>% of viral RNA</th>
<th>C_{t/2}</th>
<th>% of viral RNA</th>
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</thead>
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<tr>
<td>F 4-6 Friend cells</td>
<td>100</td>
<td>16</td>
<td>0.087</td>
<td>100</td>
<td>40</td>
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<tr>
<td>FVP-infected spleen, 20 days</td>
<td>100</td>
<td>20</td>
<td>0.070</td>
<td>100</td>
<td>65</td>
</tr>
<tr>
<td>FVP-infected spleen, 7 days</td>
<td>78.7</td>
<td>70</td>
<td>0.020</td>
<td>NT*</td>
<td>NT</td>
</tr>
<tr>
<td>FVA-infected spleen, 20 days</td>
<td>92.8</td>
<td>60</td>
<td>0.023</td>
<td>98.4</td>
<td>180</td>
</tr>
<tr>
<td>FVA-infected spleen, 7 days</td>
<td>33</td>
<td>600</td>
<td>0.0023</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Normal spleen</td>
<td>38.6</td>
<td>600</td>
<td>0.0023</td>
<td>40.5</td>
<td>1300</td>
</tr>
<tr>
<td>FVP-infected bone marrow, 20 days</td>
<td>72</td>
<td>400</td>
<td>0.0035</td>
<td>86</td>
<td>500</td>
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<tr>
<td>FVP-infected bone marrow, 7 days</td>
<td>31.9</td>
<td>500</td>
<td>0.0028</td>
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<td>NT</td>
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<td>FVA-infected bone marrow, 20 days</td>
<td>54.6</td>
<td>1800</td>
<td>0.0008</td>
<td>48</td>
<td>2500</td>
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<tr>
<td>FVA-infected bone marrow, 7 days</td>
<td>23.5</td>
<td>2000</td>
<td>0.0007</td>
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<td>NT</td>
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<td>Normal bone marrow</td>
<td>29.3</td>
<td>2000</td>
<td>0.0007</td>
<td>42.8</td>
<td>2600</td>
</tr>
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</table>

\* Maximum hybridization was 90% of input cDNA FVP to cytoplasmic RNA extracted from F 4-6 Friend cells.

\* The C_{t/2} values indicate the C_{t} value (in mol x time in seconds/liter) required to reach 50% of the final hybridization value.

\* Values indicate the amount of total cytoplasmic RNA hybridizable to the cDNA probe indicated prepared from the Friend virus complex as described in "Materials and Methods."

\* Maximum hybridization was 75% of the input cDNA SFFV\* specific to cytoplasmic RNA extracted from F 4-6 Friend cells.

\* NT, not tested.

**Chart 4.** Expression of FVP- and SFFV\* specific sequences in spleen and marrow cells at 20 days after FVA infection. Cytoplasmic RNAs from spleen (C) and marrow (O) were hybridized with FVP cDNA (top) and SFFV\* cDNA (bottom).

Results in Chart 5 relate to hybridization studies with FVP probe. The same level of viral RNA (0.0145%) is detected in GM colonies and bursts from spleen of FVP-infected mice (Chart 5, top). A similar pattern is noticed for corresponding marrow colonies (Chart 5, bottom). On the other hand, borderline levels of hybridization have been apparently observed when testing RNA extracted from bursts and GM colonies from uninfected spleen and marrow (Chart 5).

Similar results have been obtained by hybridizing RNA from bursts and GM colonies with SFFV\* probe (Chart 6). SFFV\* specific RNA is present in splenic GM colonies and bursts at levels comparable to those detected with FVP probe (0.0043%), whereas relatively low amounts are detected in marrow colonies. SFFV\* specific RNA is low or undetectable in GM colonies from uninfected spleen and marrow.
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Chart 6. Expression of SFFVp-specific sequences in cytoplasmic RNA extracted from P-BFU-E and GM colonies from spleen and marrow of mice infected for 20 days with FVP (O, P-BFU-E; A, CFU-GM) and normal animals (Δ, CFU-GM). Top, spleen colonies; bottom, marrow colonies.

DISCUSSION

The identity of target cell(s) for FV is still uncertain. Studies by Hankins et al. (11, 12) and Kost et al. (15) suggest that M-BFU-E and in part CFU-E may serve as targets. Indeed, in vitro FVP infection of normal hemopoietic cells induces growth of erythroid bursts in absence of exogenous EP. These colonies are apparently generated by M-BFU-E and to a small extent by CFU-E. In vitro FVA infection also causes formation of bursts largely derived from M-BFU-E; full hemoglobinization of these colonies, however, requires EP addition. These studies indicate that normal P-BFU-E can hardly be infected in vitro by FV. However, Axelrad et al. (1) suggested that only cycling BFU-E may serve as targets for FV. In this regard, normal P-BFU-E are largely out of the cycle (7, 33), while their proliferative rate is markedly enhanced in FV-treated mice (26). It follows that, although normal P-BFU-E are not infected in vitro by FV, the cycling P-BFU-E in infected animals may serve as targets for FV. In this regard, FV treatment perturbs the kinetics of P-BFU-E (26) and renders their growth partially BPA-independent (24). These phenomena, although suggesting that P-BFU-E are in vivo targets for FV, may be alternatively interpreted in terms of mechanisms acting primarily on other cell populations and indirectly on BFU-E.

The hybridization studies reported here throw light on the problem of the target cell for FV. They indeed show constant presence of FVP-specific sequences in pools of P-BFU-E-derived bursts from FVP-infected animals. In vitro spread of virus infection was excluded by control experiments with anti-gp70 serum. These results therefore indicate that P-BFU-E are infected in vivo by FVP, thus supporting the contention that they serve as targets for FVP.

It is additionally shown that GM colonies from FV-infected mice contain specific sequences. Surprisingly, viral RNA levels are similar in both erythroid bursts and GM colonies. It was also observed that 90% pure granulocyte or macrophage colonies show a virtually identical hybridization pattern (results not shown). These results thus document that FV infects the early progenitors of not only erythroid but also granulocyte and macrophage lineage.

It is generally conceded that P-BFU-E are very closely related to CFU-S (23). Both are characterized by identical cell size and buoyant density (33), although P-BFU-E show a slightly more elevated cycling activity than do the virtually resting CFU-S (7). The close similarity of P-BFU-E and CFU-S, as well as the infection by FV of different types of early progenitors (P-BFU-E, CFU-GM), suggests that FV may infect primarily the CFU-S. As a remote alternative possibility, FV might independently infect P-BFU-E and CFU-GM but not CFU-S.

The present results may be considered in the light of recent in vivo studies, which show a BPA rise early after FV infection (24). The elevation is apparently mediated via mechanism of both "intrinsic" and "extrinsic" type (i.e., hypersensitivity of P-BFU-E to BPA and enhanced BPA release, respectively). These results suggest that early after FV infection, enhanced BPA release may induce a rise of the cycling activity of P-BFU-E, which would then become suitable targets for FV infection. Infected P-BFU-E may in turn acquire an enhanced sensitivity to BPA, thus further enhancing the pressure for erythropoietic differentiation.

It is apparent that these in vivo results and the hybridization data reported here are not only compatible but reciprocally strengthen and clarify each other. Both support the concept that P-BFU-E may serve in vivo as targets for FV. Lack of in vitro infection of normal P-BFU-E by FV (11, 12) may be explained in terms of their low cycling activity. Our hybridization studies further show that CFU-GM are infected by FVP. However, CSF activity is not enhanced in FV-treated mice (24) thus possibly explaining lack of marked GM proliferation in the early stage of FVP erythroleukemia. Our results may also suggest that CFU-S are infected in vivo by FV (see above). If this is indeed the case, preferential differentiation of infected stem cells into the erythropoietic pathway may be attributed to the enhanced BPA (24) and EP-like activity (8, 22, 26).

Hybridization analysis of RNA sequences in infected mice shows quantitative differences in virus expression in whole spleen and marrow. Viral sequences, measured with both FVP- and SFFV-specific probes, are more abundant in RNA extracted from spleen as compared to marrow by a 10-fold factor. This confirms that the spleen is the selective organ for both virus replication and expression of the erythroid component of the disease. The difference is also in keeping with kinetic data on the early stage of FV induced disease, indicating (a) apparent migration of P-BFU-E from marrow to spleen via peripheral blood (26) and (b) more prominent expression of BPA independence of P-BFU-E at splenic than at marrow level (24).

The spleen-marrow difference is less marked when evaluating bursts and GM colonies from the 2 organs. This may be due to the different cellular composition between whole spleen and bone marrow. Viral RNA sequences may be expressed at very elevated levels in cell population present in vivo, particularly in splein, but not in bursts and GM colonies.

RNA from both normal spleen and marrow hybridizes at low levels with both probes utilized here. These data are in line with previous studies (2) on normal hemopoietic tissues from DBA-
ss mice. It has been proposed that these virus-related sequences are involved in the regulation of normal differentiation of hematopoietic cells, although their precise function is still unknown. In line with this concept, results available so far suggest that the introduction of the SFFVp genome in susceptible hosts modulates the number and the hormonal requirement of erythroid progenitors. However, no evidence has been presented that the SFFVp genome can malignantly transform hematopoietic cells in vivo. Indeed, spleen cells in the early phase of the FVP disease, although infected, have little self-renewal or proliferative capacity, are not tumorigenic, and cannot be established as cell lines (19). In view of these observations, it is interesting to speculate that the mechanism of SFFVp action may be different from the mechanism of transformation proposed for other acute leukemia or sarcoma viruses. Viruses like Harvey sarcoma virus and Abelson leukemia virus in fact seem to act by producing a transforming protein having the enzymatic activity of a protein kinase (6, 29, 34). These viruses, besides inducing neoplastic transformation in vivo, can transform fibroblasts in vitro, a feature which is not possessed by SFFVp.

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REFERENCES


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