The *Fv*-2 Gene Controls Induction of Erythroid Burst Formation by Friend Virus Infection *in vitro*: Studies of Growth Regulators and Viral Replication

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**SUMMARY**

When infected *in vitro* with Friend virus complex, the bone marrow cells of susceptible mice form large colonies (bursts) of erythroblasts after 5 days of culture in semi-solid medium. This virus-induced burst growth occurs without the addition of erythropoietin (EP) which is normally required for erythroid progenitor growth *in vitro*. Erythroid progenitor cells from C57BL/6 mice infected *in vitro* with Friend virus are resistant to virus-induced burst growth, while cells from the B6.S mouse strain, which is congenic with C57BL/6 but possesses the ‘Friend virus sensitivity’ alleles at the *Fv*-2 locus, are susceptible. This susceptibility of the B6.S cells demonstrates that virus-induced burst growth is regulated by the *Fv*-2 gene. Two mechanisms by which the *Fv*-2 locus could control virus resistance were analysed. The possible modulation of the erythroproliferative effect of the virus by soluble substances which either promote burst growth in the sensitive strains or inhibit growth in the resistant strain was examined. Also, the possible restriction of virus infection or replication in resistant (*Fv*-2*) haemopoietic cells was investigated. In a variety of experimental conditions designed to test the effects of soluble growth promoters on bone marrow cells infected *in vitro*, the resistance of C57BL/6 cells to erythroid burst formation could not be overcome. Neither could resistance be transferred to co-cultured sensitive cells by any soluble substances produced in culture by C57BL/6 cells. Use of haemopoietic cells from C57BL/6 animals in various physiological states of haemopoiesis also did not overcome the resistance to virus-induced burst growth. Quantification of several parameters of viral replication in whole marrow cultures or in erythroblasts from bursts of the *Fv*-2 sensitive and *Fv*-2 resistant congenic mouse strains showed that haemopoietic cells of both strains support virus growth equally well. These data suggest that *Fv*-2*-mediated* resistance to the erythroproliferative effect of Friend virus infection *in vitro* is an inherent property of an erythroid progenitor target cell and is not determined by external factors. The resistance is also not due to restriction of virus replication.

**INTRODUCTION**

Several lines of evidence suggest that Friend virus-induced erythroid burst formation (Hankins *et al.*, 1978) represents *in vitro* the same type of cellular event which is responsible for the rapid erythroblastosis characteristic of Friend disease in mice. Following infection *in vitro* with the polycythaemia-inducing strain of Friend virus (FVP), the 4 to 6 day period required for complete maturation of erythroid cells in virus-induced bursts is the same length of time required *in vivo* for the first increases in reticulocyte count and $^{59}$Fe uptake in the spleen (Tambourin & Wendling, 1971; Hankins *et al.*, 1979). Furthermore, the cells in the bursts induced *in vitro* by FVP or by the anaemia-inducing strain of Friend virus (FVA) differentiate to the same degree as their *in vivo* counterparts. In FVP-infected mice, the virus-induced...
erythroblastosis is accompanied by profound rapid increases in blood erythrocytes, while in FVA-infected mice the erythroblastosis is not accompanied by these dramatic erythrocyte increases (Tambourin et al., 1973). In tissue culture without added erythropoietin (EP), FVP-infected marrow cells yield bursts of erythroblasts which undergo complete differentiation into erythrocytes, while FVA-infected marrow cells yield bursts in which very few of the erythroblasts synthesize haemoglobin or undergo complete differentiation (Hankins & Troxler, 1980).

Several mouse genes greatly affect the sensitivity or resistance to Friend disease (Teich et al., 1982). One gene which has been studied extensively in terms of disease resistance is the Fv-2 locus (Axelrad et al., 1972; Odaka, 1970; Steeves et al., 1978). Hankins & Luna (1982) have shown that FVP does not induce EP-independent erythroid burst growth after infection of marrow cells taken from C57BL/6 mice that are resistant to Friend disease (Fv-2'). The present study shows that EP-independent burst growth occurs with FVP-infected cells of the B6.S mouse strain, a strain which is congenic with C57BL/6 but which possesses the Friend virus sensitivity alleles (Fv-2') at the Fv-2 locus (Axelrad et al., 1972). Thus, the Fv-2 gene or a closely linked gene has a major role in controlling induction of burst formation by the virus.

One possible mechanism for the Fv-2-mediated resistance to Friend virus is that erythroid growth regulatory substances other than EP are involved. Substances which promote growth of the early erythroid progenitors, the burst forming units-erythroid (BFU-e) (Axelrad et al., 1973), have been identified (Johnson & Metcalf, 1977; Iscove, 1978). Such burst-promoting activity (BPA) may be insufficiently produced or have insufficient effect in marrow cell cultures of Fv-2 mice. Alternatively, a soluble macromolecule that suppresses the passage of BFU-e through the cell cycle and presumably affects their progression through the erythroid differentiation pathway has been described in Fv-2' mice (Axelrad et al., 1981). This suppressor substance may somehow prevent the erythroproliferative effects of Friend virus. The present study employs various alterations in the system of virus infection in vitro with subsequent burst growth and in the physiological state of the mice providing the cells for this system, in order to test the effects of growth regulators and precursor cell physiology on Friend virus-induced erythroid burst growth.

Another possible reason that Fv-2' erythroid cells are resistant to the effects of Friend virus is failure of the virus to infect or replicate in these cells. Friend virus is a retrovirus complex consisting of a replication-defective spleen focus-forming virus (SFFV) and a replication-competent murine leukaemia virus (F-MuLV) (Troxler et al., 1980). The SFFV component is responsible for the massive erythroblastosis which occurs 2 to 3 weeks after infection of mice (Troxler et al., 1980) and also for erythroid burst formation after infection of marrow cells in vitro and subsequent culture (Hankins et al., 1978). Much less SFFV is recovered from the spleens of infected C57BL/6 mice than from infected sensitive mice (Steeves et al., 1978; Odaka, 1967). It has also been reported that SFFV replication does not persist in long-term bone marrow cultures of C57BL cells (Teich & Dexter, 1979). Nevertheless, because SFFV is replication-defective, its loss during growth in cells of Fv-2' animals may be because erythroid cells infected with this virus component would have no great selective growth advantage as compared to those from sensitive mice. Friend virus has been shown to replicate in fibroblasts from C57BL/6 mice (Evans et al., 1980), but quantitative titres of infectious virus were not measured for both disease-sensitive and -resistant strains. The study of Yoosook et al. (1980) suggested that the Fv-2 locus does not affect infectious virus replication in vitro since bone marrow cells from DBA/2 and D2. Fv-2' mice produce similar titres of virus in culture. Likewise, the results of Odaka (1970) suggest that viral replication occurs in both DDD and DDD(Fv-2') mice. However, these latter studies were not conclusive about whether Fv-2 affects virus replication quantitatively. In view of the possibility that virus replication could be decreased in erythroid cells but not in other cell types of Fv-2' mice, quantitative analyses of virus replication were done in the present study.

**METHODS**

*Mice.* (BALB/c × DBA/2)F1 mice (CD2F1) and C57BL/6N mice were obtained from the National Cancer Institute (Bethesda, Md., U.S.A.). Breeder C57BL/6UT and B6.S mice were obtained through the courtesy of Dr A. Axelrad (University of Toronto). Except when noted, marrow cells were obtained from mice with phenyl-hydrazine-induced anaemia at 8 to 10 weeks of age (Kost et al., 1979).
**Virus and virus assays.** Stock Friend virus consisted of infectious BALB/c mouse plasma. The original virus was obtained in 1968 from Robert Holdenreid at the National Institutes of Health and has been maintained by serial passage of infectious plasma in BALB/c mice since that time. The virus stock contains an NB-tropic F-MuLV at a titre of 1 × 10^7 XC plaque-forming units per ml (Rowe et al., 1970), and the polycythaemia-inducing variant of SFFV (SFFVPv) at a titre of 1 × 10^7 focus-forming units per ml (Axelrad & Steeves, 1964).

**Virus-induced erythroid bursts.** Conditions of infection of mouse bone marrow cells with Friend virus and for growth of erythroid bursts in semi-solid medium containing 0.8% methylcellulose were as described by Kost et al. (1981). Sheep plasma erythropoietin (4 units/mg protein; Step 3, Connaught Laboratories, Swiftwater, Pa., U.S.A.) was added to some cultures of uninfected control marrow cells at a final concentration of 2 units/ml. For enumeration of erythroid bursts, the cultures were clotted after 120 h and stained with 3,3'-dimethoxybenzidine diluted three- to fivefold, and the cells were recovered by low-speed centrifugation. In cases where only the cells in erythroid bursts were analysed, the bursts were plucked from the cultures at 120 h using a dissecting microscope and a modified Pasteur pipette. Populations of such plucked cells were always at least 90% erythroblasts as determined by Wright-stained cytocentrifuge preparations.

**Culture of Friend virus-infected lymphocytes.** For analysis of virus replication in cell populations consisting primarily of lymphocytes, spleen cells from normal mice were infected in vitro with Friend virus (Kost et al., 1981) and subsequently cultured at 1 × 10^6 cells/ml in the presence of 12 μg/ml of bacterial endotoxin (Escherichia coli O55 : B5, Sigma) in RPMI 1640 medium plus 10% foetal bovine serum (Alberto et al., 1982). After 4 days of culture at 37 °C in 5% CO2 in air, the cells were removed by centrifugation, and the SFFV titre in the supernatant medium was determined.

**Preparation and characterization of lectin-stimulated spleen cell-conditioned media.** Pokeweed mitogen-stimulated spleen cell-conditioned medium (PWCM), prepared essentially by the method of Johnson & Metcalf (1977), were used as sources of BPA. Briefly, 2 × 10^6 mouse spleen cells per ml were incubated for 4 days in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum and 15 μg of pokeweed mitogen per ml (Gibco). The cells were then removed by centrifugation, and the supernatant was sterilized by filtration. The PWCM was tested and shown to have the following properties which have been previously reported (Iscove, 1978). The PWCM increased by fivefold the number of erythroid bursts at 7 or 8 days in normal uninfected bone marrow cultures containing EP. When PWCM was present in the culture medium, but EP was withheld until day 4, bursts still appeared and matured on days 7 to 8 just as if EP had been present from the time of culture initiation. However, EP had to be added by day 4 or the colonies failed to enlarge further or to differentiate into recognizable erythroid cells. The BPA in the PWCM prepared from CD2F1 spleen cells supported C57BL/6 erythroid bursts equally as well as PWCM from C57BL/6 spleen cells supported CD2F1, erythroid bursts. The smallest amount of conditioned medium that showed maximum stimulation of burst growth (usually 5 to 10% by volume) was determined for each batch of PWCM and used in all further experiments.

**Isolation of infected C57BL/6 erythroblasts.** To obtain virus-infected C57BL/6 erythroblasts, marrow cells were infected with virus, and the cells were cultured for 7 days at 4 × 10^5 cells per ml in methylcellulose culture with 2 units per ml of EP. Infected C57BL/6/6B6.S and C57BL/6/6B6.S were at comparable stages of differentiation in terms of morphology and haemoglobin content.

**Analysis of virus-specific RNAs.** Total RNA of erythroblasts or of total cells from bone marrow cultures was extracted with hot phenol and SDS (Scherrer & Darnell, 1962). Formaldehyde–agarose gel electrophoresis of RNA and ethidium bromide staining of the gels was as previously described (Rave et al., 1979). Blotting of the RNA onto nitrocellulose sheets and subsequent hybridization was by the method of Thomas (1980) except that no dextran sulphate was included in the hybridization solution. The virus-specific DNA probe which was used in the hybridization was labelled by nick translation (Rigby et al., 1977) with [α^32P]dCTP (800 Ci/mmol) to a specific activity of approximately 1 × 10^8 c.p.m./μg of DNA. The probe was a recombinant pBR322 plasmid containing the complete genome of F-MuLV, originally cloned into bacteriophage lambda by Oliff et al. (1980). In addition to the gel blot analysis, aliquots of RNA were diluted in 3 M-NaCl, 0.3 M-sodium citrate (pH 7.0) and applied directly to nitrocellulose paper by filtration in a HYBRI-DOT apparatus (Bethesda Research Laboratories).

**Analysis of synthesis of proteins.** For analysis of globin polypeptide synthesis, plucked erythroblasts were incubated for 2 h in leucine-free Eagle's MEM containing 50 μCi/ml [3H]leucine (20 Ci/mmol) and 20% (v/v) dialysed foetal bovine serum. Stroma-free cell lysates were separated electrophoretically on urea/Triton X-100/acyclic acid polyacrylamide gels (Alter & Goff, 1980).

**Analysis of synthetic rates of viral proteins** was conducted by pulse-labelling cells at 1 × 10^6 cells/ml for 2 h in methionine-free MEM containing 50 μCi/ml [35S]methionine and 20% (v/v) dialysed foetal bovine serum. After labelling, the cells were lysed in radioimmunoprecipitation assay buffer (Witte et al., 1979). Aliquots containing 1 × 10^6 c.p.m. of 35S were incubated first with non-immune goat serum, and antigen–antibody complexes were removed by precipitation with Staphylococcus aureus (Kessler, 1975). The aliquots were then reacted with goat antiserum prepared against the purified envelope glycoprotein, gp70, of a xenotropic murine retrovirus.
RESULTS

Role of the Fv-2 gene and of erythroid burst-promoting activity on Friend virus-induced erythroid burst growth

Fig. 1 shows that Friend virus-induced erythroid burst formation occurred only in marrow cultures originated from CD2F1 mice or B6.S mice but not in cultures from C57BL/6 mice. The formation of bursts in B6.S but not C57BL/6 cultures indicates that the Fv-2 locus plays a decisive role in the process since these mouse strains are congenic except at that locus. In numerous experiments, the B6.S mice always produced only one-third to one-half as many viral bursts per 10^6 plated cells as the CD2F1 strain, but the bursts which did develop were as large as those of CD2F1. Careful microscopic examination of virus-infected C57BL/6 marrow cultures in situ with an inverted microscope and when clotted and stained with 3,3'-dimethoxybenzidine and haematoxylin failed to reveal any evidence of erythroid colony or burst development. The resistance of C57BL/6 marrow cells to virus-induced erythroid burst formation was not overcome by the addition of PWCM to the cultures. Fig. 1 shows that the addition of PWCM to Friend virus-infected marrow cultures of sensitive CD2F1 mice increases the number of erythroid bursts which are induced by the virus. The bursts are also larger in virus-infected CD2F1 cultures containing PWCM. These results suggest that cell-derived growth factors are beneficial and perhaps essential for development of bursts in response to virus. However, the data of Fig. 1 demonstrate that PWCM alone without virus infection cannot cause burst development by the mouse marrow cells.

Attempts to render Fv-2rr cells sensitive to virus in vitro

A wide variety of experiments were done to render C57BL/6 erythroid precursors sensitive to the in vitro effects of Friend virus. Extensive washing of marrow cells from C57BL/6 mice with DMEM [a procedure which removes the negative cell cycle regulator described by Axelrad et al. (1981)] before Friend virus infection and culturing in vitro did not result in virus-induced erythroid burst formation. It was reasoned that perhaps in C57BL/6 marrow, very few erythroid precursors are normally at the developmental stage of the mature burst-forming unit erythroid (BFU-e) which is the main target of the virus in vitro (Kost et al., 1981). Therefore, an attempt was made to generate mature BFU-e in vitro by culturing the marrow cells for 2 or 3 days in the presence of PWCM before infection. Since PWCM supported BFU-e development in place of EP for the first 3 to 4 days of the BFU-e assay (see Methods), normal marrow cells were cultured in methylcellulose medium for 2 or 3 days with PWCM, then washed, infected with Friend virus and recultured for 5 days in methylcellulose medium containing PWCM. No virus-induced burst formation was seen in C57BL/6 cultures (Table 1). Control cultures to which EP, instead of virus, was added in secondary culture contained numerous fully haemoglobinized, EP-induced bursts by 5 days after re-plating. This control showed that there were many erythroid precursors present after the 2 days of primary culture which were at a similar stage of differentiation as the target cells for Friend virus infection in vitro (Kost et al., 1981). Similar experiments using marrow cells of CD2F1 mice yielded large numbers of bursts upon infection with Friend virus after primary culture with PWCM and re-plating in methylcellulose secondary cultures. The average of 56 bursts per 1.25 × 10^5 infected CD2F1 cells (Table 1) is about four times the number of virus-induced bursts formed by infected primary marrow cells in the presence of PWCM (Fig. 1), indicating that generation or enrichment of viral targets occurred during the initial culture of CD2F1 cells with PWCM. Thus, C57BL/6 marrow cells cannot be rendered sensitive to Friend virus-induced erythroid burst formation by washing, by addition of PWCM, or by prior culture in the presence of PWCM.

In addition to the above mentioned in vitro manipulations of the cells, numerous attempts were made to manipulate the erythropoietic state of the C57BL/6 animals prior to taking the
Friend virus and erythropoiesis

Fig. 1. Mouse strain susceptibility to virus-induced erythroid burst growth: effect of PWCM. Aliquots (0.5 ml) of methylcellulose medium containing the indicated number of cells were plated in 16 mm wells. ○, Infected CD2F1 cells without conditioned medium; ●, infected CD2F1 cells with PWCM; □, infected C57BL/6 cells with or without PWCM; ■, uninfected CD2F1 cells with or without PWCM; △, infected B6.S cells without PWCM; ▲, infected B6.S cells with PWCM. Data are means ± 1 standard deviation of triplicate cultures.

Table 1. Resistance of C57BL/6 marrow cells to virus-induced erythroid burst formation

<table>
<thead>
<tr>
<th>Origin of marrow cells (strain)*</th>
<th>Primary culture in PWCM</th>
<th>Burst-inducing agent during secondary culture</th>
<th>Number of erythroid bursts after 5 days of secondary culture†</th>
</tr>
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<tbody>
<tr>
<td>CD2F1</td>
<td>+</td>
<td>SFFV</td>
<td>56 ± 8</td>
</tr>
<tr>
<td>CD2F1</td>
<td>+</td>
<td>EP</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CD2F1</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>+</td>
<td>SFFV</td>
<td>0</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>+</td>
<td>EP</td>
<td>&gt;100</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>+</td>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cells (10⁶ per ml) were cultured for 2 days in methylcellulose medium with PWCM, then removed for experimental treatment. After removal from primary culture, the cells were infected with Friend virus and re-plated (2.5 × 10⁵ cells per ml) or were re-plated without infection in the presence or absence of 2 units/ml of EP. The medium of the secondary culture also contained PWCM. Identical results were obtained whether the PWCM was prepared from CD2F1 or C57BL/6 spleen cells or whether the primary culture period was for 2 or 3 days.
† After 5 days of secondary culture, the cultures were clotted and stained with 3,3'-dimethoxybenzidine and haematoxylin for observation of erythroid bursts. Numbers are means of bursts for quadruplicate, 0.5 ml aliquots (1.25 × 10⁵ cells) plated in 16 mm plastic wells ± 1 standard deviation.

Attempts were made to demonstrate virus-induced bursts in cultures of in vitro infected cells from 13-, 14- and 15-day foetal mouse livers, newborn to 6 week-old mouse bone marrows or spleens, regenerating marrow from mice treated with an LD₅₀ dose of 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) and unit-gravity separation gradients of extensively washed cells from spleen and marrow of phenylhydrazine-treated animals. A high percentage of BFU-e obtained from C57BL/6 animals under most of these conditions have been shown to be supporting DNA synthesis, as determined by [³H]thymidine or hydroxyurea suicide experiments (Suzuki & Axelrad, 1980; Koury & Krantz, 1982). No virus-induced burst induction was noted with C57BL/6 spleen cells obtained under any of these circumstances. With CD2F₁ cells, the foetal and newborn cell sources were excellent for virus-induced burst formation, providing about twofold more targets on a per cell basis than marrow cells directly
Fig. 2. Globin synthesis in virus-induced erythroid bursts from mixed cultures of CD2F1 and C57BL/6 cells. (a) [3H]Leucine pulse-labelled globin polypeptides synthesized by uninfected CD2F1 erythroblasts from bursts arising in marrow cultures incubated in the presence of 2 units per ml of EP for 7 days. The erythroid bursts were plucked, labelled, and analysed as described in Methods. (c) The globin pattern for similarly prepared C57BL/6 erythroid burst cells. (b) The globin pattern for erythroid bursts which resulted from viral infection of a 1:1 mixture of CD2F1 and C57BL/6 cells. The virus-induced bursts were plucked at 120 h of culture for pulse-labelling with [3H]leucine. α, α globin; βmajor and βminor, β globin chains of CD2F1 mice; βsingle is the β globin chain type of C57BL/6 mice. All lanes contained approximately equal amounts of radioactivity (10⁶ c.p.m.), but (b) was exposed 20 times longer than (a) or (c) to demonstrate the lack of βsingle synthesis in the mixed culture.

from adult animals treated with phenylhydrazine. Cells from regenerating or immature mouse sources were equivalent to their counterparts in normal adult mice (8 to 13 viral bursts per 10⁶ cells). These data indicate that the phase of the cell cycle of the erythroid progenitor cells at the time of isolation and infection does not account for the resistance of C57BL/6 erythroid cells to burst formation after Friend virus infection in vitro.

Growth of virus-induced bursts in mixed populations of sensitive and resistant marrow cells

Possibly, C57BL/6 marrow cells elaborate suppressor substances within the cultures which prevent Friend virus-induced erythroid burst growth in vitro. To test this possibility a 1:1 mixed population of CD2F1 and C57BL/6 marrow cells was infected with Friend virus and cultured in methylcellulose medium without EP. At 120 h, the mixed cultures contained about one-half as many virus-induced erythroid bursts as control cultures containing an equal number of purely CD2F1 cells. These results were consistent with only the sensitive cell type producing bursts. To show that the bursts in mixed cultures came only from the cells of the CD2F1 strain, the bursts were analysed for globin chain type. Fig. 2 shows the results of a globin analysis in bursts grown in mixed CD2F1 and C57BL/6 cell populations. The detectable β globins are βmajor and βminor.
Table 2. *Infectious virus production by C57BL/6, B6.S and CD2F1 cells*

<table>
<thead>
<tr>
<th>Infected cells</th>
<th>SFFV titre (f.f.u./10^6 cells)</th>
<th>F-MuLV titre (p.f.u./10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1 (marrow cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>100</td>
<td>2600</td>
</tr>
<tr>
<td>CD2F1</td>
<td>500</td>
<td>3300</td>
</tr>
<tr>
<td>B6.S</td>
<td>120</td>
<td>2300</td>
</tr>
<tr>
<td>Expt. 2 (marrow cells)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>140</td>
<td>2600</td>
</tr>
<tr>
<td>CD2F1</td>
<td>700</td>
<td>3500</td>
</tr>
<tr>
<td>B6.S</td>
<td>180</td>
<td>2800</td>
</tr>
<tr>
<td>Expt. 3 (endotoxin-treated spleen lymphocytes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>CD2F1</td>
<td>600</td>
<td></td>
</tr>
</tbody>
</table>

* Supernatants from infected cells were removed at 5 days post-infection for marrow cultures and at 4 days for lymphocyte cultures.

which are produced by CD2F1 cells. Even at long exposure times, the β single produced by C57BL/6 mice was not visible. Therefore, the bursts induced in the mixed cultures were from CD2F1 cells and not C57BL/6 cells. Identical results were obtained when PWCM was added at the initiation of culture. Co-cultivation experiments were also done using infected C57BL/6 and B6.S marrow cells. In these experiments the number of bursts at 120 h was consistent with burst formation only by B6.S cells. Thus, no suppressive soluble factor produced in vitro by C57BL/6 cells can be acting under the conditions of these cultures.

**Virus replication in C57BL/6 marrow cells in culture**

To determine whether or not C57BL/6 haemopoietic cells in culture produce as much infectious virus as cells of the sensitive strains, titres of released virus were examined. Bone marrow cells from CD2F1, B6.S and C57BL/6 mice were infected and cultured in methylcellulose medium. After 5 days, the semi-solid medium was diluted with an equal volume of DMEM, the cells were sedimented and the supernatants were analysed for infectious F-MuLV and for SFFV. The cell numbers were determined at the end of the culture period so that titres could be expressed on a per cell basis. The titres of F-MuLV were similar in the supernatants from the three strains (Table 2). SFFV was produced at significant levels in all cultures; however, the SFFV production was not equal in the three strains. SFFV production per cell in CD2F1 cultures was about fivefold higher than in C57BL/6 cultures or B6.S cultures. Cell culture supernatants from endotoxin-stimulated, infected spleen lymphocytes were also assayed after 4 days of culture to compare SFFV production by non-erythroid cells of CD2F1 and C57BL/6 mice (Table 2). Again, a fivefold difference in SFFV production in the lymphoid cultures of the two strains indicated that the higher SFFV production in CD2F1 marrow cultures is not restricted to erythroid cells which predominate in those cultures.

**Viral RNA production**

Quantitative analysis of viral RNA production in cultured cells was performed in order to try to answer two questions: (i) why is SFFV production lower in marrow cultures of C57BL/6 and B6.S strains than in those of the CD2F1 strain and (ii) are the cells of the erythroid lineage in C57BL/6 mice specifically resistant to virus infection? RNA was extracted from total populations of infected marrow cells after 5 days in methylcellulose culture. Both the electrophoretic blot analysis and the dot blot analysis (Fig. 3) show that there were approximately equal amounts of viral RNAs in the CD2F1 and C57BL/6 cells relative to the quantities of rRNAs. In fact, in the experiment shown in Fig. 3, C57BL/6 cells had slightly more viral RNAs than CD2F1 cells. For example, lanes 2' and 5' of Fig. 3(c) show that 20 μl aliquots of the C57BL/6 and CD2F1 RNAs contain about the same amounts of total rRNA. Yet the C57BL/6 RNA gave a slightly stronger hybridization signal (lane 2, Fig. 3(a)) than CD2F1 RNA.
Fig. 3. Viral RNA synthesis in infected CD2F1 and C57BL/6 marrow cells in culture. Total cellular RNA from $10^6$ cells was dissolved in 10 µl of electrophoresis buffer. (a) Nitrocellulose blot of a formaldehyde-agarose gel which was hybridized with $^{32}$P-labelled plasmid DNA containing the genome of F-MuLV. Lanes 1 and 2 of (a) contained 10 µl and 20 µl, respectively, of RNA from infected C57BL/6 cells while lanes 3 to 6 contained 5 µl, 10 µl, 20 µl, and 30 µl of RNA from infected CD2F1 cells. (b) Autoradiograph of hybridized dot blots of serial dilutions containing 2 µl, 5 µl, 10 µl, and 20 µl of the same preparations of C57BL/6 (C57) and CD2F1 (CD2) RNAs as (a). (c) Photograph of the ethidium bromide-stained gel of (a). (d) Autoradiograph of a hybridized filter containing RNA from uninfected cells which was exposed 40 times longer than (a).

(lane 5, Fig. 3a). Likewise, the dot blots (Fig. 3b) show that for comparable amounts of RNA, the C57BL/6 preparation gave a just slightly stronger signal. The ratios by weight of SFFV to F-MuLV genomic RNAs, determined by cutting out the bands and counting in a scintillation counter, were 1:3 to 1:5 in the whole marrow cultures from both mouse strains. Fig. 3(d) shows a hybridized blot of cellular RNAs from uninfected cells. The lanes contained amounts of RNA comparable to lanes 2' and 5' of Fig. 3(e) (about 10 µg). To visualize the bands in the Fig. 3(d) autoradiograph, it was necessary to expose it for 160 h as opposed to 4 h for those shown in Fig. 3(a). The bands of 28S and 18S apparently represent non-specific adherence of labelled DNA to the rRNAs in the lanes. The band marked by an asterisk in the CD2 lane of Fig. 3(d), which probably represents transcripts of an endogenous retrovirus sequence, is intermediate in size between the genomic bands of F-MuLV and SFFV in Fig. 3(a). RNA from infected B6.S cells
Fig. 4. Viral RNA synthesis in erythroblasts plucked from cultures as described in Methods. Five μg samples of total cell RNA were subjected to formaldehyde-agarose electrophoresis and blot hybridization with 3²P-labelled F-MuLV DNA and an autoradiograph was made. Lane 1, C57BL/6 erythroblast RNA; lane 2, C57BL/6 RNA from cells remaining in culture after removal of erythroid bursts; lane 3, CD2F₁, erythroblast RNA from virus-induced erythroid bursts. 28S and 18S positions mark the locations of the rRNAs detected by staining of the gel with ethidium bromide.

was also analysed (although not shown in Fig. 3) and contained virus-specific species which were qualitatively and quantitatively the same as for the other two strains.

To test whether or not erythroid cells of C57BL/6 mice are specifically resistant to virus infection, the accumulation of intracellular, viral genomic RNA in plucked C57BL/6 and CD2F₁ erythroblasts was examined (Fig. 4). To get virus-infected bursts in C57BL/6 cultures, it was necessary to add EP to the medium and to plate the marrow cells at 4.0 × 10⁵ cells/ml, which is one-fifth the density used for virus-induced burst growth. Lane 1 shows RNA extracted from infected C57BL/6 erythroblasts and lane 2 shows RNA from the remaining cells in a similar culture after the bursts were plucked. The cells represented by lane 2 were mostly granulocytes and macrophages and contained few erythroid cells. In the C57BL/6 erythroblasts and other cells as well, the ratio of SFFV to F-MuLV RNAs is about 0.5 compared to the value of 1.3 seen in Fig. 3. This reduced ratio is due to less efficient SFFV spread in the low density C57BL/6 cultures as compared to the higher density cultures depicted in Fig. 3 because similar cultures of CD2F₁ and B6.S marrow cells infected and plated at 4.0 × 10⁵ cells/ml also exhibited ratios of SFFV to F-MuLV RNAs approaching the lower value of 0.5 (not shown). Other experiments showed that the ratio of SFFV to F-MuLV RNAs did not change between days 5 and 7 in C57BL/6 or CD2F₁ cultures, demonstrating that the later harvesting of C57BL/6 bursts compared to CD2F₁ bursts in this study did not influence the conclusions. The similarity of lanes 1 and 2 of Fig. 3 indicates that the erythroid cells exhibited no specific restriction to SFFV infection. Fig. 4, lane 3 shows the RNA from infected CD2F₁ erythroblasts isolated by plucking virus-induced bursts from low density culture. The high RNA ratio of SFFV to F-MuLV (1.5) reflects the fact that all virus-induced bursts must arise from cells infected with SFFV regardless of the culture density.
Production of the SFFV-encoded protein

Evidence from several studies (for review, see Coffin, 1982) suggests that an SFFV-encoded glycoprotein of approximate molecular weight 52000 (gp52) is responsible for the erythropoietic effects of Friend virus. gp52 is a hybrid viral protein containing a substantial portion of the viral envelope protein, gp70. Virus-infected erythroblasts from CD2F$_1$ and C57BL/6 bursts were examined for production of proteins related to the viral gp70. Fig. 5 (lanes 2 and 3) shows that the major protein immunoprecipitated from infected C57BL/6 and CD2F$_1$ erythroblasts with anti-BALB : virus-2 gp70 antiserum has a molecular weight of about 50000. The next most abundant labelled proteins had molecular weights of about 82000 and 70000. These proteins were also specifically precipitated with antisera to Rauscher MuLV gp70 and to whole disrupted Friend MuLV (not shown). Control erythroblasts from uninfected, Ep-induced bursts treated with anti-gp70 serum (lane 1) or infected erythroblasts treated with normal goat serum (lane 4) were negative for these proteins. Due to their specific precipitation with antisera and their molecular weights, these proteins most likely represent gp52, gp70, and the precursor of
gp70 (molecular weight 82000) some of which remains unprocessed during the 2 h labelling time. Although the amount of gp52 appears to be less in C57BL/6 erythroblasts than in erythroblasts from virus-induced CD2F₁ bursts, this again is consistent with the fact that the C57BL/6 erythroblasts were isolated from low density culture with EP and that some of them are probably not infected. Thus, there does not appear to be any significant reduction of gp52 in infected C57BL/6 cells. Cells from virus-induced B6. S bursts appeared to be equivalent to CD2F₁ burst cells in gp52 content (not shown).

**DISCUSSION**

The Fv-2 gene controls susceptibility of mice to the acute erythroblastosis of Friend disease. The present study shows that this gene locus also determines susceptibility of mouse cells to Friend virus-induced erythroid burst growth in vitro. In an attempt to elucidate how Fv-2 regulates virus-induced burst growth, the roles of soluble growth-regulatory substances and virus replication were examined in sensitive and resistant cells.

Soluble growth-promoting factors are released by many cell types in vitro and in vivo, and they promote development of early erythroid progenitors in culture without addition of exogenous EP (Johnson & Metcalf, 1977; Iscove, 1978). The existence of such factors raises the possibility that the early polycythaemia induced in mice by Friend virus and the analogous in vitro induction of erythroid burst growth in semi-solid culture medium (Hankins et al., 1978) could be the result of an indirect effect of the virus on non-erythroid cells which in turn release soluble erythroid growth factors. In this study, PWCM containing demonstrable BPA increased the number of virus-induced bursts observed in CD2F₁ and B6. S marrow cultures (Fig. 1), and it made linear the relation between number of viral bursts observed and the number of infected cells plated. However, PWCM could not cause burst development in the CD2F₁ marrow cultures without virus infection, and it could not promote virus-induced burst formation in C57BL/6 marrow cells. Thus, soluble burst-promoting factors are not capable of mimicking Friend virus in causing EP-independent burst development. This conclusion is further supported by the results of Fig. 2, which show that infection of a 1 : 1 mixture of washed marrow cells from CD2F₁ and C57BL/6 mice leads to burst formation exclusively by CD2F₁ progenitors. Any effective 'trans'-acting burst-promoting factors secreted into the medium by auxiliary cells should have produced C57BL/6 erythroid bursts in the mixed population experiments.

The production of virus-induced erythroid bursts in marrow cultures from B6. S mice (Fig. 1) shows that viral burst production is controlled by the Fv-2 gene or a closely linked gene, since B6. S and C57BL/6 mice are isogenic except at that locus (Axelrad et al., 1972). Axelrad et al. (1981) have provided evidence that Fv-2 controls production of a soluble suppressor of cell proliferation which acts on erythroid progenitors. However, the experiments in Table 1 and Fig. 2 argue against a simple role for this suppressive regulatory factor as the mediator of Fv-2-determined Friend virus resistance in vitro. Extensive washing of C57BL/6 cells to remove a soluble regulator did not render the erythroid progenitors sensitive to Friend virus; neither did incubation of the marrow cells for 2 or 3 days with PWCM (Table 1), a procedure which maintains developing erythroid precursors within the cultures. It is possible that a negative regulatory factor is regenerated quickly after washing and infection, thus negating the viral effect in these experiments. However, the C57BL/6 cells in the mixed culture (Fig. 2) did not produce a soluble regulator that prevented development of CD2F₁ bursts within the same culture. Likewise, no inhibition of virus-induced B6. S bursts was seen when C57BL/6 and B6. S cells were mixed, infected, and cultured. These in vitro cocultivation experiments confirm results obtained in vivo with radiation chimeras by Silver & Teich (1981), which showed that Fv-2sr erythroid cells are susceptible to viral effects in an animal of Fv-2sr genotype and in the presence of other Fv-2sr marrow precursors. Additional evidence suggests that the fraction of erythroid progenitors in the S phase of the cell cycle does not control susceptibility to virus effects. Increased percentages of erythroid progenitor cells (BFU-e) from C57BL/6 mice have been previously shown to be in DNA synthesis in the spleen and bone marrow of animals under 6 weeks of age (Suzuki & Axelrad, 1980), and also in regenerating marrow after treatment with BCNU (Koury & Krantz,
induction by Friend virus also does not appear to depend on the phase of the cell cycle of the progenitor BFU-e. Resistance to in vitro burst induction by Friend virus also does not appear to depend on the phase of the cell cycle of the progenitor BFU-e.

Several aspects of viral replication were examined quantitatively in C57BL/6, CD2F1, and B6. S cells. The results of the virus-specific RNA analyses (Fig. 3 and 4) indicate that there is no restriction of F-MuLV or SFFV penetration or early replication stages (viral genomic RNA production) in C57BL/6 cells. This appears to be true in the whole marrow cultures and specifically in infected erythroblasts. Immunoprecipitation of pulse-labelled viral envelope-related proteins from infected C57BL/6 and CD2F1 erythroblasts (Fig. 5) shows that both produce gp52 and gp70 precursors. Furthermore, the levels of production of these proteins as well as other viral proteins (not shown) are similar in the two strains. The release of the helper F-MuLV (Table 2) was similar in cultures of all three strains. The release of infectious SFFV was the same in C57BL/6 and B6. S cells but it was about fivefold greater in CD2F1 cells. Although the genetic basis for this more efficient SFFV production by CD2F1 cells is unknown, it is not related to the Fv-2 gene since C57BL/6 and B6. S had similar titres of SFFV. Also, this genetic difference does not appear to be cell type-specific since reduced SFFV production was evident in C57BL/6 lymphocyte cultures as well as marrow cultures. The differences in infectious SFFV titres result from a viral replication stage beyond RNA synthesis. The RNA comparisons are the most accurate of the viral quantification measurements presented here because of the internal standard provided by rRNAs. All of the data indicate that Fv-2-mediated resistance to virus-induced erythroid proliferation is not due to lack of replication of SFFV in Fv-2+ cells.

The data presented here demonstrate that the Fv-2 genetic locus or some closely linked locus controls the ability of erythroid progenitor cells infected in vitro with Friend virus to proliferate in response to the virus. The Fv-2-mediated susceptibility of these cells to the proliferative effects of the virus cannot be attributed to externally acting soluble regulators of erythroid growth. Rather, susceptibility to Friend virus' effects appears to be an inherent property of the infected erythroblast progenitor. Furthermore, the data support the notion that this inherent Fv-2-mediated susceptibility is not related directly to virus replication or production within an erythroid progenitor but rather is related to the intracellular response of the progenitor to the virus infection.

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REFERENCES


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