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Erythroid colony induction without erythropoietin by Friend leukemia virus *in vitro*

(Friend spleen focus-forming virus/erythropoiesis/plasma culture/erythroleukemia)

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ABSTRACT **Erythroid colonies could be produced without the addition of erythropoietin in plasma cultures seeded with bone marrow cells from normal C3Hf/Bi mice by exposure of the cells *in vitro* to medium from a cell line (IS) that continuously produces Friend leukemia virus in culture. The activity in the culture medium was viral rather than erythropoietin-like, since it was sedimentable by high-speed centrifugation and heat labile. Erythroid colonies did not develop when the bone marrow cells exposed to virus-containing medium were from mice genetically resistant to Friend virus. IS culture medium contained both Friend spleen focus-forming and XC-plaque-forming activities. No erythroid colonies were induced when genetically sensitive cells were exposed to a preparation from which the spleen focus-forming activity had been removed, but which contained XC plaque-forming activity in high concentration. Thus the spleen focus-forming component of Friend virus appeared to be responsible for inducing erythroid colony formation without erythropoietin *in vitro*. Some erythroid colonies were also found in control cultures to which neither virus nor erythropoietin had been added. Reduction in the concentration of fetal calf serum in the culture medium substantially decreased the number of these colonies but had only a minor effect on the number of virus-induced colonies. The number of erythroid colonies produced after 2 days of culture without erythropoietin or fetal calf serum was approximately proportional to the titer of Friend spleen focus-forming virus to which the bone marrow cells had been exposed. This system should prove useful for investigation *in vitro* of Friend virus–host cell interactions which lead to erythropoietin-independent erythropoiesis.**

Infection of susceptible mice with certain preparations of Friend leukemia virus (FV) (1) rapidly leads to an erythroid disease (2–4) in which erythropoiesis appears to proceed in the absence of stimulation by erythropoietin (Epo) (5, 6), the normal humoral regulator of erythroid cell proliferation and differentiation. Studies in plasma culture have revealed that following FV infection *in vivo*, a massive change occurs in the erythropoietic cell population of the host: normal colony-forming units that respond to Epo [referred to as CFU-E (7, 8)] are rapidly replaced by colony-forming units capable of producing erythroid colonies *in vitro* in the absence of Epo (9).

Friend virus preparations may contain two distinctly different viral activities. One of these is the ability to induce erythroid disease, a function unique to Friend and related virus preparations, and correlated with their content of a virus (Friend SFFV), which induces macroscopic foci in the spleens of susceptible hosts (10). A second is the ability to induce the formation of syncytia and macroscopic plaques in cultures of XC cells [Rous virus-transformed rat nonproducer cells (11)], a function shared with many murine leukemia viruses incapable of inducing erythroid disease. These two viral activities can be assayed separately (12, 13). The relation between them is still not clear.

Analysis of cellular and viral events in the development of erythroid disease induced by FV preparations would be greatly facilitated if a system were available in which transformation of the hemopoietic cell population could be brought about *in vitro* under controlled conditions. We, therefore, attempted to devise such a system. Since acquisition of the capacity for erythropoietin-independent erythroid colony formation is a prominent feature of the condition induced by FV *in vivo*, we directed our attention to this particular function as an indicator of significant viral action *in vitro*. Two innovations proved advantageous: (*1*) use of FV-containing medium from a chronically infected continuous cell line growing *in vitro*, rather than virus from the plasma of FV-infected mice, and (2) omission of Epo from the media in which bone marrow cells were infected and cultivated.

We now report the induction of erythroid colony formation without Epo by bone marrow cells infected *in vitro* with a preparation of FV, and present evidence that the virus responsible for the effect is Friend SFFV.

MATERIALS AND METHODS

Mice. Female C3Hf/BiUt, C57BL/6Ut, and SIM/Ut mice, 8–12 weeks old, were from the Division of Laboratory Animal Science, University of Toronto.

Virus. The Friend leukemia virus used to infect normal bone marrow cells *in vitro* was harvested from the medium of a continuous cell line IS, clone B3 (Clarke *et al.*, manuscript in preparation) derived from the spleen of a SIM mouse infected with a polycythemia-inducing strain of Friend virus (9). Friend SFFV titers were determined by the spleen focus assay method (12) and are expressed in focus-forming units (FFU)/ml, and Friend XC plaque-forming virus titers were determined according to Rowe *et al.* (13) on secondary mouse embryo culture from SIM strain mice and are expressed in plaque-forming units (PFU)/ml. IS cells were grown in Falcon tissue culture dishes (Falcon Plastics, Oxnard, Calif.) in α modified minimum essential medium supplemented with 10 μg/ml of the eight nucleosides and 10% unheated fetal calf serum (FCS) (Flow Laboratories, Rockville, Md.).

Infection and Cell Culture. Collection and plasma culture of bone marrow cells and the assay method for erythropoietin-dependent CFU-E have been described previously (8). Unless otherwise noted, C3Hf/BiUt bone marrow cells were used in all experiments.

Infection of bone marrow cells *in vitro* was performed by mixing 0.2 ml of collection medium (2% heat-inactivated

Abbreviations: Epo, erythropoietin; CFU-E, colony-forming unit that responds to Epo; FV, Friend virus; SFFV, spleen focus-forming virus; FFU, focus-forming unit; PFU, plaque-forming unit; FCS, fetal calf serum.

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fetal calf serum) containing 4×10^6 pooled nucleated bone marrow cells with either 0.2 ml fresh chilled medium from a subconfluent 3- to 5-day ISB3 culture or 0.2 ml of fresh control medium containing 10% FCS. Final concentration of FCS during infection was therefore 6%. The mixture was then placed in an ice-bath with shaking every 15 min. After 45 min 0.2 ml of bovine embryo extract, 0.2 ml of bovine serum albumin, 0.2 ml of L-asparagine, 0.4 ml of FCS, 0.4 ml of NCTC-109, and 0.2 ml of bovine plasma were added to the mixture to give a final volume of 2.0 ml. The concentrations of each of these constituents were as previously described (8). Aliquots of 0.1 ml volume were quickly dispensed into six individual microtiter wells which were then incubated at 36–37° in an atmosphere of 5% CO_2 and high humidity. Colonies of eight or more benzidine–peroxide staining cells were scored at 48 hr. The plasma culture system normally contains 20% FCS (0.4 ml in 2.0 ml) but in later experiments FCS was omitted and was replaced by medium NCTC-109. The volume of fresh IS or α culture medium added to each plasma culture was 0.01 ml and, together with the medium in which the cells were suspended, yielded a final concentration of 1.2% FCS in virus-infected and control plasma cultures.

RESULTS

Effect of FV-containing medium on erythroid colony formation in plasma cultures without erythropoietin

Bone marrow cells were treated with media from IS cell cultures or control medium and plated in plasma culture. Epo was not included so that the search for any erythroid colonies that formed in the absence of added Epo could be made in primary cultures. After 2 days' incubation, erythroid colonies were observed, and their numbers were found to be 2- to 3-fold greater in the IS medium-treated cultures than in controls. The colonies in the treated cultures were indistinguishable morphologically from those produced following exposure of hemopoietic cells to Epo (8). No "erythropoietic bursts" (14) were observed in the cultures of bone marrow cells exposed to IS medium.

Effect of centrifugation and temperature

If the induction of erythroid colonies by IS culture medium was brought about by a virus in the medium rather than by a soluble agent such as an Epo-like factor, then the supernatant of IS culture medium subjected to high-speed centrifugation should not induce appreciable numbers of erythroid colonies and the activity should be recoverable from the pellet. Accordingly, medium from IS cells was centrifuged at

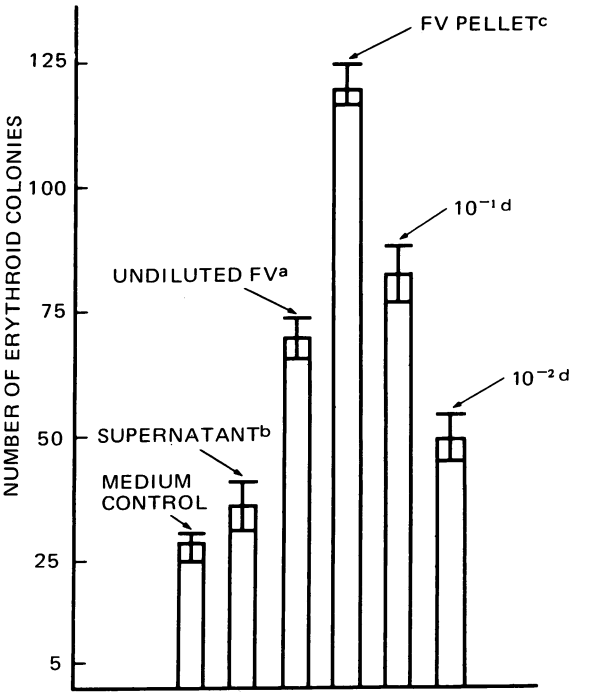

FIG. 1. Effect of concentration of FV-containing medium on erythroid colony formation *in vitro*. (a) Medium from ISB3 roller bottle culture (passage 40) harvested at 10 days after trypsinization and plating. The volume of undiluted, uncentrifuged medium added to each plasma culture contained 80 FFU of spleen focus-forming Friend virus as titrated *in vivo* in SIM mice. (b) Particles removed from medium by centrifugation at 100,000 × *g*, 1 hr. The volume of supernatant added to each culture contained <2 FFU of Friend spleen focus-forming virus. (c) Resuspended pellet of ISB3 medium. The volume added to each plasma culture contained 6000 FFU of spleen focus-forming Friend virus; (d) Dilutions of pelleted FV. Error bars indicate SEM.

100,000 × *g* for 1 hr. Both the supernatant and the resuspended pellet were assayed for their ability to induce erythroid colonies in the absence of added Epo *in vitro* and for their content of Friend SFFV *in vivo*. Fig. 1 illustrates that the undiluted IS medium yielded more than twice as many erythroid colonies as the control; the activity in the IS medium could be removed by centrifugation, and it could be recovered from the pellet in concentrated form. Moreover, the number of erythroid colonies produced *in vitro* was related to the Friend SFFV titer as assayed *in vivo*. The capacity of IS culture medium to induce the production of erythroid

Table 1. Effect of heating FV-containing medium on erythroid colony formation in plasma cultures without erythropoietin

Treatment	Friend spleen focus-forming virus content of medium (FFU/culture)*	Concentration of medium added to cultures	No. of erythroid colonies†
FV-containing medium‡	70	Undiluted (0.01 ml)	174.6 ± 12.5
FV-containing medium‡	—	10^{-1} (0.01 ml)	23.0 ± 2.6
FV-containing medium‡	—	10^{-2} (0.01 ml)	15.0 ± 1.5
FV-containing medium‡	—	10^{-3} (0.01 ml)	16.3 ± 2.0
Heated§ FV-containing medium‡	<0.2	Undiluted	15.8 ± 2.7

* Titrated *in vivo* in random-bred Swiss females.
† Bone marrow cells (2×10^5) plated per culture; 0% FCS. Numbers are mean ± SEM.
‡ Medium passed through 0.45 μm Millipore filter before addition to cultures.
§ 56° for 30 min.

Table 2. Effect of strain of origin of bone marrow cells on induction of erythroid colony formation by FV-containing medium in plasma cultures* without erythropoietin

Treatment	No. of cells	No. of erythroid colonies		
		C3Hf/Bi	SIM	C57BL/6 (B6)
Medium control	2×10^5	30.5 ± 3.1	18.3 ± 1.9	11.8 ± 1.1
FV-containing medium	2×10^5	131.2 ± 5.0	36.3 ± 3.3	12.5 ± 1.4
Erythropoietin	5×10^4	231.5 ± 9.1	205.5 ± 8.9	208.6 ± 14.0

* With 20% FCS.

colonies by bone marrow cells in plasma cultures without Epo was evidently due to its content of a particulate entity, which, like Friend SFFV, could be sedimented by centrifugation.

Murine leukemia viruses rapidly lose their biological activity when exposed to elevated temperatures (15). To determine whether the erythroid colony-inducing activity was heat labile, FV-containing IS medium was passed through a 0.45 μm pore-size Millipore filter and subjected to a temperature of 56° for 30 min. Its capacity to induce erythroid colonies in plasma culture without Epo and to induce spleen foci *in vivo* were then assessed. Table 1 shows that both the capacity of IS culture medium to induce erythroid colonies and its Friend SFFV titer were lost after heating. This result is consistent with the hypothesis that the ability of IS culture medium to induce erythroid colonies was due to a virus and not to Epo, since the latter is known to be extremely heat-stable (16). The data in Table 1 also show that the number of erythroid colonies produced fell to background levels when the Friend SFFV titer was between 7 and 0.7 FFU per culture.

Effect of strain of origin of bone marrow cells

The sensitivity of murine cells to FV is under strict genetic control. Mice of the C3H/Bi and SIM strains [of genotype *Fv-1*^{nn}; *Fv-2*^{ss} (17–19)] are known to be highly sensitive to the spleen focus-forming activity of N-tropic FV. Mice of the C57BL/6 (B6) strain [of genotype *Fv-1*^{bb}; *Fv-2*^{rr} (18)] are solidly resistant to the spleen focus-forming activity of FV, irrespective of its tropism (20). The FV produced by IS cells is N-tropic. To determine whether or not host genotype influenced the production of erythroid colonies in cultures without Epo, bone marrow cells from C3Hf/Bi, SIM, and C57BL/6 mice were exposed to IS culture medium and plated in plasma cultures. Controls received α medium only. Cells from the various strains, which had not been exposed to IS culture medium *in vitro*, were also cultured in the presence of Epo.

Table 2 demonstrates that while Epo induced the formation of erythroid colonies by bone marrow cells independently of their strain of origin, the medium from IS cultures induced the formation of erythroid colonies in cultures without Epo only when mixed with bone marrow cells of C3H and SIM strain mice, but not with those of the B6 strain. This result indicates that the phenomenon of erythroid colony induction by IS culture medium is genetically controlled in a manner similar to that of FV and strongly suggests that the active entity in IS culture medium is FV itself.

Effect of fetal calf serum concentration

In many experiments, where 2×10^5 cells were plated per 0.1 ml of culture, control values in the absence of added Epo were high and thus obscured the effect of FV on erythroid colony formation. If fetal calf serum normally present at a concentration of 20% in the plasma culture medium were responsible for the high backgrounds, elimination of this constituent might increase the resolution of the system.

Bone marrow cells exposed to FV-containing IS culture medium were plated in cultures containing various dilutions of FCS in medium NCTC-109. Table 3 shows that reduction of the FCS concentration from 20 to 0% (actually 1.2% due to residual serum from FV-containing medium) strikingly lowered the number of erythroid colonies in control cultures but had much less effect on the number of erythroid colonies in FV-infected cultures. This made it possible to detect a greater than 10-fold increase in the number of erythroid colonies induced by the virus (Table 3). Assays for FV-induced erythroid colonies in plasma culture were thereafter routinely performed in the absence of added FCS.

Effect of XC plaque-forming virus in FV-containing medium

Preparations of FV are known to contain at least two viral activities, XC plaque-forming activity (11, 13) and spleen focus-forming activity (12). XC plaque-forming virus is regularly found in excess of spleen focus-forming virus in various FV preparations; it has therefore proved possible to obtain XC plaque-forming virus free of spleen focus-forming virus by end-point dilution (21). Moreover, XC plaque-forming virus replicates well in mouse embryo cultures (13).

Table 3. Effect of fetal calf serum concentration on FV-induced erythroid colony formation in plasma cultures without erythropoietin

Treatment	No. of cells	No. of erythroid colonies			
		20% FCS	10% FCS	5% FCS	0% FCS
Medium control*	2×10^5	128.0 ± 7.3	63.6 ± 3.0	17.3 ± 4.2	9.5 ± 2.9
FV-containing medium*	2×10^5	314.9 ± 6.5	195.4 ± 18.6	216.2 ± 8.5	166.6 ± 7.9
Erythropoietin	5×10^4	184.0 ± 7.9	166.3 ± 8.7	119.5 ± 10.9	110.2 ± 10.5

* Filtered through 0.45 μm Millipore filter.

Table 4. Effect of XC plaque-forming virus in FV-containing medium on the induction of erythroid colonies in plasma cultures without erythropoietin

	No. of cells	PFU/0.1 ml of culture†	FFU/0.1 ml of culture‡	No. of erythroid colonies
Medium control*	2×10^5	—	—	36.8 ± 3.5
FV-containing medium*	2×10^5	470	100–200	87.8 ± 10.6
FV-containing medium* passaged on mouse embryo culture	2×10^5	770	<2	25.0 ± 3.0
Erythropoietin§	5×10^4	—	—	130.8 ± 10.0

* Filtered through 0.45 μm Millipore filter. Plasma cultures contained 0% FCS.
† XC-plaque assay on SIM secondary mouse embryo culture.
‡ Titrated *in vivo* in random-bred Swiss females.
§ Standard assay for CFU-E in plasma cultures containing 20% FCS.

The Friend virus released by IS cells contains XC plaque-forming virus and SFFV in ratios of PFU/ml: FFU/ml which have varied over the range 5:1 to 100:1 (Clarke *et al.*, manuscript in preparation). To determine whether or not the XC plaque-forming virus of IS culture medium was sufficient to induce erythroid colonies in plasma culture, the FV-containing medium from IS cells was diluted to 10^{-2} and used to infect a continuous cloned SIM mouse embryo cell line. The cells were passaged twice and the 4-day culture medium was assayed for PFU, FFU, and the ability to induce erythroid colonies in plasma culture without Epo or FCS. It is evident from Table 4 that, following passage of diluted virus from IS cells in mouse embryo culture, the medium had a high titer of PFU and a low titer of FFU. Infection of bone marrow cells with the passaged virus preparation, despite its high PFU titer, did not induce erythroid colony formation in plasma cultures without Epo. It thus appears that the presence of Friend spleen focus-forming virus activity is necessary for erythroid colony induction by FV *in vitro*. The high colony number in controls, low numbers of CFU-E, and relatively low efficiency of the virus in this experiment are unexplained.

Effect of variation in dose of FV-containing IS culture medium on number of erythroid colonies in plasma cultures without Epo

To determine the relation between the dose of FV-containing IS culture medium and the number of erythroid colonies produced, various dilutions of the virus-containing medium and a control medium were mixed with known numbers of normal bone marrow cells and plated in plasma cultures containing no added Epo or FCS. After 2 days' incubation the numbers of erythroid colonies were determined. The IS culture medium was also assayed for spleen focus-forming activity *in vivo*.

Fig. 2 shows the results of two experiments in which the number of erythroid colonies produced at 2 days in cultures without Epo is plotted against the dilution of IS culture medium used for infection (abscissa below). The zero point on the abscissa below is used to designate the medium control. The Friend SFFV titer of undiluted IS culture medium (FFU/0.1 ml of culture) as determined *in vivo* is recorded at the right-hand end of the abscissa above on each graph, and an appropriate dilution scale is included.

The number of erythroid colonies produced was approximately proportional to the concentration of IS culture medium. It is also evident that the capacity of IS culture medium to induce the formation of erythroid colonies *in vitro* diluted out at about the same level as did its capacity to induce spleen foci *in vivo*. The observations are consistent with the notion that a single entity is limiting for the induction of each erythroid colony and that this entity is probably identical with the Friend spleen focus-forming virus.

When the concentration of virus-containing medium was fixed at 80% or 100% of undiluted medium (48–65 FFU) and the number of bone marrow cells exposed to the virus was varied, the erythroid colony-inducing activity was found to dilute out at around 5×10^4 to 1×10^5 bone marrow cells per 0.1 ml of culture.

DISCUSSION

Cells of bone marrow exposed to Friend spleen focus-forming virus *in vitro* rapidly developed into erythroid colonies in cultures containing no added Epo. This phenomenon is almost certainly the counterpart *in vitro* of Epo-independent erythroid colony formation in cultures of hemopoietic cells from animals infected *in vivo* (9). Its significance in relation to the regulation of erythropoietic differentiation and to the induction of erythroid disease has been discussed (9).

As the basis of an enumerative response assay method for one "transforming" function of Friend SFFV, erythroid colony induction in cultures without Epo has certain advantages and limitations. It is quantitative [the number of erythroid colonies induced by the virus appeared to be roughly proportional to, and of the same order of magnitude as, the number of FFU of Friend SFFV used for infection (Fig. 2)]. It is reasonably sensitive, since the lowest dose of virus at

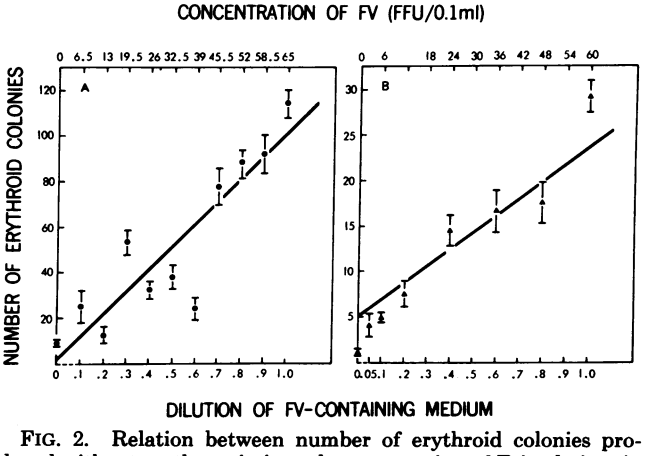

FIG. 2. Relation between number of erythroid colonies produced without erythropoietin and concentration of Friend virus in medium used for infection *in vitro*. A least-squares fit of y vs x for six curve types showed that for both curves A and B the data were best fitted by a straight line. The intercepts on the y-axis (2.5, 3.0) were not significantly different from the control values ($P > 0.2$; $P > 0.1$).

which the number of induced erythroid colonies became significantly greater than background was around 5–20 FFU per culture. Since this amount of virus was contained in a volume of 0.01 ml at the time of infection, the minimum virus titer required to register in this system was 5×10^2 to 2×10^3 FFU/ml. It is rapid (2 days *in vitro* as opposed to 9 days *in vivo*), and it is inexpensive (a pool of marrow cells from three mice suffices for a full titration with six microtiter wells per point, each holding only 0.1 ml of culture medium as opposed to a minimum of six mice per point in the spleen focus assay method). However, factors still unknown and uncontrolled introduce substantial uncertainties into the data. The most important factor limiting the usefulness of the method at present is that so far, it is restricted to assays of Friend SFFV from IS cell cultures.

Less than 1–2 FFU of the virus (as measured *in vivo*) induced the production of one erythroid colony *in vitro* among 2×10^5 bone marrow cells (Fig. 2). Normal bone marrow cell populations are known to be highly heterogeneous and it is likely that only a minority of the cells could have served as potential targets for the virus. While a figure for the multiplicity of infection will have to await determination of the actual number of target cells in murine bone marrow, the data already indicate that, under the conditions of these experiments, the probability that a virus–cell interaction will occur in this system and lead to erythroid colony induction must be high. The findings also emphasize the fact that the micro-environment of bone marrow or spleen is not necessary for this action of the virus.

Thus one of the requirements for a Friend leukemia virus transformation system *in vitro* has been fulfilled by the present work. It remains to be determined whether other aspects of the leukemic transformation can also be brought about *in vitro* by FV—e.g., increase in the total number of erythroid colony-forming units (9), production of TCFU [which give rise to macroscopic colonies in the spleens of mice genetically resistant to the virus but histocompatible with cells of the donor strain (22, 23)], and continuous growth *in vitro* (24).

The source of Friend SFFV used in this system was a continuous line of cells, IS clone B3, that chronically released virus while growing *in vitro* (Clarke *et al.*, manuscript in preparation). Virus from the plasma of FV-infected animals was found to induce few or no erythroid colonies *in vitro* with FFU doses at which virus from IS cultures was highly effective. Whether this indicates a genetic difference between these viruses or a difference in their physiological condition deserves further investigation. Differences in the ratios of XC plaque-forming virus to SFFV do not seem to be involved, since preparations of FV from the plasma of infected mice and of virus from IS cells are similar in this respect.

Omission of fetal calf serum from the culture system eliminated a major source of possible contamination with trace amounts of Epo. This had the important practical effect of reducing the number of background colonies and so improved the resolution of the viral induction system. However, the fact that the efficiency of erythroid colony formation following infection was only slightly impaired raises the possibility that the virus may have also imposed another property on the cells it affected—a reduced dependence on factor(s) in fetal calf serum required for their growth and differentiation [cf. (25)].

The erythroid colonies induced by the virus were morphologically indistinguishable from those produced by normal CFU-Es in response to Epo and never exceeded the latter in number. In both, differentiation proceeded as far as the production of erythrocytes. In addition, both types of erythroid colonies were fully developed within 2 days of culture, after which lysis set in (8). These observations suggest that both Friend SFFV and Epo may affect the same target cells. If so, competition between the two agents for a limited number of these targets *in vitro* could explain why the presence of Epo in the cultures did not facilitate the induction of erythroid colonies by virus, as might have been expected from data obtained *in vivo* (26, 9).

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