A Quantitative Spleen Colony Assay Method for Tumor Cells Induced by Friend Leukemia Virus Infection in Mice

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SUMMARY

A quantitative spleen colony method is described for assay ing tumor cells in suspensions from the tissues of mice infected with Friend leukemia virus (FV). The method depends upon the use of (C3H × C57BL)F1 hybrid mice as hosts. These animals are able to accept tumor cell transplants from parental strain C3H mice but are resistant to the spleen focus-inducing action of FV, which may be brought in with the tumor cells to be assayed.

Discrete, macroscopically visible colonies were regularly formed in the spleens of normal F1 hybrid hosts within 9 days after the intravenous injection of FV-infected C3H spleen cells. Evidence is presented which establishes the fact that these spleen colonies resulted from the neoplastic proliferation of injected cells; they were not induced in the host by virus. The mean number of colonies formed per spleen was found to be directly proportional to the number of nucleated cells injected. Each colony was thus derived from a single entity; this provides the basis for the assay of FV-induced tumor cells in terms of tumor colony-forming units (TCFU).

FV-induced tumor cells could also be assayed on the basis of spleen colony formation in heavily irradiated mice; spleen foci cannot be induced by FV in such animals. Specific Friend antiserum incubated in vitro with infected spleen cell suspensions prior to their injection into irradiated hosts had a cyto toxic action on Friend antigen-containing cells only. This permitted a distinction to be made between FV-induced tumor colonies and normal hemopoietic colonies in the spleen. For a given FV-infected cell suspension, the content of TCFU estimated by assay in heavily irradiated mice was not significantly different from that obtained in unirradiated F1 hybrid mice.

INTRODUCTION

Within days following the infection of susceptible mice by Friend leukemia virus (FV), new cells appear in the spleen. Their unregulated proliferation leads to the production of either discrete foci or massive, diffuse spleen enlargement, depending on the conditions under which infection occurred; both are characteristic of the early disease induced by this virus (3, 17, 18, 30). Late in the course of Friend virus infection, cells have been demonstrated in the spleen which have the capacity for continuous proliferation when transplanted into normal hosts or cultivated in vitro (8, 16, 19, 20, 34). But whether the infected cells acquire these neoplastic characteristics late in the disease or whether tumor cells already exist during the early phases of infection and form an integral part of the new cell population that arises in response to the virus is still an open question. Because of their inherent interest for studies on virus-induced abnormality of growth regulation and because so little is known about their properties, we felt that it would be valuable to try to devise an assay method for investigating these early cells quantitatively.

The spleen colony method seemed an obvious choice in view of its successful application in quantitative studies on lymphoma (5–7, 9) and plasma cell tumor populations (D. E. Bergsagel and F. A. Valeriote, in preparation). This method was based on the observation (7) that, following the intravenous injection of neoplastic (but not normal) cell suspensions into normal, genetically compatible mice, discrete colonies developed in the spleen. These colonies, which were large enough to be visible to the naked eye 8–12 days after injection, could be shown to be derived from the extensive proliferation of donor cells that had settled in the normal host spleen and could thus be regarded as tumor colonies. Since the number of tumor colonies formed per spleen was found to be directly proportional to the number of nucleated cells injected, counts of these colonies could be used to obtain a direct measure of the number of tumor colony-forming units (TCFU) in the injected cell suspension, no matter how heterogeneous its cell population, and without the necessity for any assumptions regarding morphologic properties of the tumor cells responsible for colony formation.

It was not unreasonable to expect that if the spleens of mice, recently infected with virus, contained tumor cells, these cells might also form colonies in the spleens of normal hosts. However, it was evident from the beginning of the present study that a difficulty existed here which was not encountered with the lymphomas or with the plasma cell tumor. Cell suspensions from Friend virus-infected mice may contain large quantities of virus capable of rapidly inducing foci in the spleens of susceptible host mice; the virus-induced foci might then be indistinguishable from tumor colonies resulting from the proliferation of the injected cells. Confusion between the actions of
virus and cells in the spleen might thus render it impossible to use the spleen colony method for detecting tumor cells in Friend virus-infected cell suspensions. If, however, spleen focus induction by Friend virus could be prevented in some way, it should then become feasible to detect the presence of any colonies that formed by proliferation of the injected cells in the spleens of normal hosts.

In the work to be described, a genetic and a radiobiologic procedure were used to inhibit spleen focus induction by Friend virus. This made it possible to demonstrate that certain cells in early Friend virus-infected spleen suspensions are, in fact, capable of giving rise to tumor colonies in the spleen. The proportional relationship found between the number of colonies thus produced and the number of nucleated cells injected forms the basis of a quantitative assay method for tumor cells in any Friend virus-infected cell suspension.

MATTERIAlS AND METHODS

**Materials**. The mice used in these experiments were C3H/HeNOci (C3H/HeNOci x C57BL/6J)c1 F1, (C3H/HeNOci x C57BL/6J)c1 F1, and their reciprocal hybrids, bred in the animal colony of the Ontario Cancer Institute.

Six- to 12-week-old C3H and F1 hybrid mice were housed in metal cages and supplied freely with Rockland Mills Mouse Diet, (Teklad Inc., Monmouth, Ill.) and water. Randomized mice of the same sex were used for each experiment.

**Irradiation of Mice.** Irradiation of mice was carried out in the 137Cs unit described by Cunningham et al. (12). The dose rate was approximately 115 rads/min. Dosimetry was based on absorbed dose measurements with a Fricke dosimeter (40).

**Medium.** The standard medium used in this study was Krebs-Ringer-Phosphate (KRP) medium (14) containing glucose (5.0 gm/liter), gelatin (2.5 gm/liter) (24), penicillin (10,000 units/ml), and streptomycin (0.1 gm/liter). In certain of the virus preparations, KRP alone was used instead of the standard medium.

**Virus.** Friend leukemia virus originally obtained from the American Type Culture Collection in 1963 and maintained in C3H/HeNOci was used. Virus stocks were routinely made from C3H mice which had been injected with high doses of FV 9-18 days earlier. The stocks consisted of either filtered supernatants of centrifuged homogenates of infected spleens, prepared by the method of Gross (21), as previously described (3), or resuspended pellets obtained from the high-speed centrifugation of infected plasma according to de Harven and Friend (13). Virus preparations were stored in liquid nitrogen.

**Virus Assays.** The infectivity of FV was titrated by the spleen focus assay method of Axelrad and Steeves (3). Known dilutions of virus preparations from plasma or spleen extracts in 0.5 ml of standard medium were injected i.v. into groups of 6 to 10 mice. Nine days later, the mice were sacrificed, the spleens were fixed in Bouin’s fluid, and the foci on the surface of each spleen were counted with the naked eye. Virus titers were expressed in focus-forming units (FFU)/ml, as determined in C3H mice. (One FFU is that amount of virus required to induce the formation of 1 focus per spleen, on the average, in a specified host strain).

**Preparation and Assay of Spleen Cell Suspensions.** Spleen cell suspensions were prepared from pools of 3-4 spleens from either normal or FV-infected C3H mice. For the FV-infected spleens, the mice were infected 7-14 days earlier with 100-1250 FFU of FV. The whole spleen was minced with scissors and the cells, together with ice-cold medium, were passed through a coarse mesh (110/inch) stainless steel screen (Greening Wire Company, Hamilton). Aliquots of the cell suspensions were counted in a hemocytometer. Data given are based on total nucleated cell counts. FV-infected spleen cells were routinely washed twice in medium to remove free virus before being injected.

A 0.5 ml volume of appropriately diluted spleen cell suspension was injected i.v. by tail vein into each of a group of assay mice. Nine days (unless otherwise stated) after the injection, the mice were sacrificed and the spleens were fixed in Bouin’s fluid. Discrete, circumscribed yellow lesions larger than 0.5 mm in size on the surface of the spleen were counted with the naked eye.

**Freezing and Thawing of Cell Suspensions.** Freezing and thawing was used to break up intact cells in infected C3H spleen suspensions. The suspension in a plastic tube was quick-frozen in a mixture of dry ice and 95% alcohol and was thawed in warm running tap water.

**Irradiation of Infected Spleen Cells.** FV-infected spleen cells were irradiated in a 137Cs unit at a dose rate of approximately 115 rads/min. Appropriate dilutions of washed, infected spleen cell suspensions were irradiated in plastic tubes surrounded by ice in the irradiation chamber. Dosimetry was measured as previously described (40).

**Preparation of Antisera.** For use as antigens, FV-infected spleen cell suspensions and Gross virus-induced leukemic cell suspensions were agitated with a magnetic stirrer in 1:500 neutral phosphate-buffered formalin (26) at 4°C for 24 hours. This treatment was known to reduce FV infectivity at least 5 log units but still retain significant Friend antigenic activity (41). C3H mice were injected intraperitoneally with 107 formalinized cells at weekly intervals for 9 weeks. For the first of the 9 treatments, the formalinized cells were emulsified with an equal volume of complete Freund’s adjuvant (containing 0.5 mg/ml of heat-killed, dried mycobacteria). Mice were bled four days after the last treatment. Normal C3H serum was also collected for use as control. The sera were heated to 56°C for 30 min and stored at -10°C until the day of use. The FV-neutralizing activity of Friend antiserum was tested according to Steeves and Axelrad (41). No attempt was made to test the Gross virus-neutralizing or cytotoxic activity of the serum obtained from mice treated with Gross leukemic cells.

**Inactivation of Spleen Colony-forming Activity of Infected Spleen Cell Suspensions by Friend Antiserum.** Routinely, 1-2 x 109 infected spleen cells/ml in standard medium were mixed with undiluted Friend antiserum (at a ratio of 1:1 to 3:1 by volume) and incubated at 37°C for 30 min. In one experiment, guinea pig serum (Connaught Medical Research Laboratories, Toronto) was added to the mixture of infected spleen cells and Friend antiserum as a source of complement. Since no significant difference was found in the degree of inactivation of spleen colony formation by antiserum with or
without complement, guinea pig serum was omitted in later experiments.

Absorption of Friend Antiserum. In the test for specificity of Friend antiserum against the tumor colony-forming activity of FV-infected spleen cells, Friend antiserum was absorbed with FV-infected spleen cells as follows. An FV-infected spleen cell suspension was washed twice in standard medium at 4°C to remove free virus. To the pellet of approximately 10^8 cells obtained after the second centrifugation were added 2 ml of Friend antiserum (diluted 1:3.2) and 1 ml of undiluted guinea pig serum. The pellet was agitated into suspension and incubated at 37°C for 30 min. The suspension was then sedimented by low-speed centrifugation in the cold and the supernatant was absorbed twice more with fresh, washed cell suspension by the same procedure. Following the final low-speed centrifugation, the supernatant was centrifuged at 30,000 × g for 1 hour (Lourdus, Model AT centrifuge with Rotor 224) at 4°C to remove any residual virus, incubated at 56°C for 30 min and stored at −10°C until used.

RESULTS

In setting out to develop a colony assay method for tumor cells induced by FV, we made use of two well-known facts. The first was that F1 hybrid mice accept transplants of cells from either parent strain. The second is that F1 hybrids, produced by mating a strain which is highly susceptible to FV with the C57BL strain, which is highly resistant, are themselves relatively resistant to FV (2, 43). Theoretically, then, if FV-infected cells from a susceptible parent strain were injected into the appropriate F1 hybrid, the spleens should remain practically free of foci induced by the virus, while colonies resulting from proliferation of the injected cells should be readily visible.

To test the applicability of this genetic approach, it was necessary to show (a) that the F1 hybrids we chose to use are indeed resistant to FV, (b) that discrete, localized lesions visible to the naked eye are produced in the spleen when FV-infected cell suspensions from the susceptible parent strain are injected into these virus-resistant hybrids, and (c) that the lesions produced are colonies due to the proliferation of the injected cells, and not foci induced in the host spleen by virus, either carried in with the injected cells or produced by these cells after they have reached the spleen.

Comparison of Susceptibility of C3H and (C3H × C57BL)F1 Hybrid Mice to the Spleen Focus-inducing Activity of Friend Virus. C3H strain mice, known to be susceptible to FV (1, 15, 31, 32), were mated to C57BL strain mice, and the appropriate F1 hybrid, the spleens should remain practically resistant to FV, (b) that discrete, localized lesions visible to the naked eye are produced in the spleen when FV-infected cell suspensions from the susceptible parent strain are injected into these virus-resistant hybrids, and (c) that the lesions produced are colonies due to the proliferation of the injected cells, and not foci induced in the host spleen by virus, either carried in with the injected cells or produced by these cells after they have reached the spleen.

Production of Discrete Macroscopic Lesions in the Spleens of F1 Hybrid Mice by FV-infected C3H Spleen Cell Suspensions and by FV: Effect of Freezing and Thawing. The following experiment was designed to determine whether localized lesions would be produced in the spleens of (C3H × C57BL)F1 hybrid mice by the i.v. injection of cell suspensions from FV-infected spleens of C3H mice, and if so, to test the effect on the production of these lesions of destroying viable cells in the suspensions.

Two dilutions of a washed FV-infected spleen cell suspension were injected into groups of 10 F1 hybrid mice. In addition, 3 groups of F1 hybrids received graded dilutions of the same cell suspension previously subjected to 3 cycles of freezing and thawing.

The data in Table 1 show that localized lesions visible to the naked eye were produced in the spleens of the F1 hybrid hosts injected with washed, FV-infected C3H cell suspension. When aliquots of the same cell suspension were subjected to 3 cycles of freezing and thawing before injection, the number of splenic lesions was reduced about 800-fold.

This reduction in the number of splenic lesions could be due to the effect of freeze-thawing on the ability of either cells, or virus, or both to produce lesions in the spleen. To determine

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td><strong>Hosts</strong></td>
</tr>
<tr>
<td><strong>C3H</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>(C3H × C57BL)F1</strong></td>
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</tbody>
</table>

Comparison of susceptibility of C3H and (C3H × C57BL)F1 hybrids to Friend virus.

*Mean numbers of foci per spleen were obtained from groups of 7 mice except in the group labeled b, where data were from 8 mice.

**Table 2**

<table>
<thead>
<tr>
<th>Number of spleen cells injected</th>
<th>Mean number of lesions per spleen ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Untreated</strong></td>
<td><strong>Frozen-thawed</strong></td>
</tr>
<tr>
<td>2 × 10^5</td>
<td>4.0 ± 1.2</td>
</tr>
<tr>
<td>2 × 10^6</td>
<td>confluent</td>
</tr>
<tr>
<td>2 × 10^7</td>
<td>0.5 ± 0.2</td>
</tr>
</tbody>
</table>

Production of discrete, macroscopic lesions in the spleens of (C3H × C57BL)F1 hybrids by FV-infected C3H spleen cell suspensions and by FV: effect of freezing and thawing. FV, Friend virus.

**Table 2**

<table>
<thead>
<tr>
<th>Dose of virus injected (focus-forming units)</th>
<th>Mean number of foci per spleen ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Untreated virus</strong></td>
<td><strong>Frozen-thawed virus</strong></td>
</tr>
<tr>
<td>0.5</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>1.5</td>
<td>1.4 ± 0.9</td>
</tr>
<tr>
<td>4.5</td>
<td>3.4 ± 2.0</td>
</tr>
</tbody>
</table>

Production of discrete, macroscopic lesions in the spleens of (C3H × C57BL)F1 hybrids by FV-infected C3H spleen cell suspensions and by FV: effect of freezing and thawing. FV, Friend virus.

*Mean numbers of lesions per spleen were obtained from groups of 10 (C3H × C57BL)F1 hybrid mice.

*Mean numbers of spleen foci were obtained from groups of 7 (C3H × C57BL)F1 hybrid mice.
Suteera Thomson and Arthur A. Axelrad

whether freeze-thawing had any effect on the focus-inducing activity of FV, a stock of FV was diluted, one aliquot was kept at 4°C, and another was rapidly frozen and thawed 3 times. The control and the frozen-thawed virus preparations were then assayed for spleen focus-inducing activity in F₁ hybrids. F₁ hybrid mice were chosen for this experiment despite their reduced susceptibility to the virus because we were interested in distinguishing between the actions of cells and virus in these hosts. Large doses of virus were used to compensate for the reduced susceptibility.

Freezing and thawing had no significant effect on the spleen focus-inducing activity of FV (Table 2). The possibility that the presence of cells might affect the sensitivity of FV to freeze-thawing was eliminated in other control experiments in which cells were deliberately added to known quantities of virus and the mixtures subjected to freezing and thawing prior to assay (unpublished data). The results of these experiments are consistent with the view that the vast majority of localized, macroscopic lesions produced in the spleens of F₁ hybrids after the injection of washed spleen cell suspensions from FV-infected C3H mice are due, not to virus, but to cells in the injected suspensions.

Effect of Ionizing Radiation on the Ability of FV-infected C3H Spleen Cell Suspensions to Produce Discrete, Macroscopic Lesions in the Spleens of F₁, Hybrids. We wished to obtain independent evidence on the question of whether cells or virus were responsible for the splenic lesions produced by the injection of FV-infected cell suspensions into normal F₁ hybrid hosts. Our plan was based on the fact that the ability of mammalian cells to carry on the sustained proliferation necessary for colony formation is a highly radiosensitive function (9, 35, 42), while FV is highly resistant to ionizing radiation (17, 25). If the focal lesions in the spleen were, in fact, colonies resulting from the proliferation of the injected cells, their production should be greatly reduced by small doses of ionizing radiation.

One to three dilutions of a washed FV-infected spleen cell suspension were exposed to different doses of ionizing radiation, and the surviving ability of the suspensions to produce discrete splenic lesions was tested in groups of 10 (C3H × C57BL)F₁ hybrids. Chart 1 shows the results obtained from three of such experiments. The radiation survival curve is evidently composed of a radiation-sensitive and radiation-resistant component. About 98% of the activity in the suspension appeared to be highly radiosensitive, with a D₀ of approximately 90 rads, a value consistent with the radiosensitivity of mammalian colony-forming cells. The D₀ of spleen focus formation by FV, 2.4 × 10⁴ rads (A. A. Axelrad and S. Thomson, to be published), is many orders of magnitude greater than this.

The remaining 2% of the activity in the suspensions was radioresistant; no significant change in survival could be observed at doses ranging from 500 to 6000 rads. This radioresistant portion of focal lesion-producing activity in the washed, infected spleen cell suspensions is thus in all likelihood due to their content of FV.

These results could be interpreted as showing that the vast majority of the discrete, macroscopic lesions in the spleens of F₁ hybrid mice injected with suspensions of FV-infected C3H spleen cells resulted from proliferation of the injected cells. However, an equally tenable hypothesis would be that the capacity of these cells to produce FV is also highly sensitive to ionizing radiation. Although F₁ hybrid mice were shown to be relatively resistant to FV (Table 1), they might conceivably be susceptible to high local concentrations of virus produced by infected cells which had been deposited in the spleen in the vicinity of sensitive target cells. In this case the splenic lesions would be foci induced by virus rather than tumor colonies produced by proliferation of the injected cells in the normal host spleen. The same argument could be raised in regard to the experiment on the effect of freeze-thawing on the production of splenic lesions. Although this showed that cells were responsible, it did not rule out the possibility that the cells acted by producing virus which in turn induced spleen foci. The induction of new spleen foci by virus thus had to be critically excluded before tumor colony formation in the spleen by FV-infected cells could be unequivocally demonstrated.

It is known that FV cannot induce the formation of foci in the spleens of heavily irradiated mice (3). The induction of spleen foci could therefore be critically excluded if heavily irradiated mice were used as hosts for the transplants of FV-infected spleen cells. Any discrete, macroscopic lesions which formed in the spleens of such mice could only be colonies resulting from the proliferation of injected cells. However, infected spleen suspensions contain normal spleen cells as well as FV-infected cells. Till and McCulloch (25, 42) have shown that hemopoietic cells in normal spleen suspensions can form macroscopically visible colonies in the spleens of irradiated mice. If normal hemopoietic colony-forming cells were present in the FV-infected spleen suspensions, they would produce colonies in the spleens of the irradiated hosts which might be indistinguishable from colonies produced by the FV-infected cells.
Old et al. (32) and Wahren (43) have demonstrated that Friend antiserum is cytotoxic to FV-induced leukemic cells when incubated in vitro in the presence of complement. If it could be shown that Friend antiserum (a) has no effect on normal hemopoietic colony-forming cells, and (b) is specifically cytotoxic to FV-infected cells, as judged by inhibition of production of the macroscopic splenic lesions, an unequivocal method would be available for detecting FV-induced tumor colony-forming cells in irradiated hosts.

Effect of Friend Antiserum on the Production of Spleen Colonies by Normal Hemopoietic Colony-forming Cells. A normal spleen cell suspension was incubated with Friend antiserum (1 ml of normal spleen cell suspension containing 10^6 cells: 0.3 ml of undiluted Friend antiserum) at 37°C for 30 minutes and then assayed in (C3H × C57BL)F1 mice previously irradiated with 900 rads. The numbers of colonies obtained with antiserum-treated and untreated control suspensions (Table 3) were not significantly different from one another. Therefore, Friend antiserum had no demonstrable effect on normal hemopoietic colony-forming cells.

Specificity of the Action of Friend Antiserum on the Production of Discrete Macroscopic Lesions in the Spleen by FV-infected Spleen Cells. To test the specificity of Friend antiserum, 2 ml aliquots containing 2 × 10^6 cells from the FV-infected spleen cell suspensions were incubated at 37°C for 30 minutes with 2 ml of (a) standard medium, (b) normal C3H serum, (c) an untreated serum obtained from C3H mice repeatedly injected with formalized Gross virus-infected leukemic cells (Gross serum), (d) Friend antiserum, (e) Friend antiserum preabsorbed with FV-infected spleen cells, and (f) absorbed Friend antiserum alone as a separate control. These suspensions were then injected into groups of 7 normal (C3H × C57BL)F1 hybrid mice. (Irradiated hosts would have been more desirable but were not used in this experiment.)

The results in Table 4 show that while Friend antiserum caused a greater than 20-fold reduction in the number of splenic lesions produced by FV-infected spleen cells, there was no detectable effect on this number by normal serum, Gross serum, or Friend antiserum previously absorbed with FV-infected cells. Friend antiserum may thus be considered to react specifically with those entities in FV-infected C3H spleen suspensions that are responsible for the production of localized macroscopic lesions in the spleens of normal F1 hybrid hosts.

Effect of Friend Antiserum on the Production of Spleen Colonies by FV-infected Spleen Cells in Normal and Heavily Irradiated Hosts. Since the spleen colony-forming activity of normal hemopoietic colony-forming cells is insensitive to Friend antiserum, any reduction in the number of colonies produced by preincubation of FV-infected spleen cells with Friend antiserum should be due to the presence in the infected cell suspension of colony-forming cells possessing FV-induced antigen. These colonies could only develop as a result of proliferation of injected cells; virus produced by or carried in with these cells would not form foci in the spleens of irradiated hosts.

Friend antiserum was added to a washed, FV-infected spleen cell suspension in the ratio 1:1, i.e., 1 ml of infected spleen cell suspension (10^6 cells/ml) to 1 ml of undiluted antiserum. The antiserum-treated suspension and an untreated control suspension were incubated at 37°C for 30 min. The two suspensions were then assayed in unirradiated and irradiated F1 hybrids. The results are shown in Table 5. Friend antiserum had a pronounced effect in reducing the number of splenic lesions produced by the infected spleen cell suspensions in all groups.

Most striking is the finding that the absolute reduction in the number of splenic lesions due to the antiserum was the same in irradiated (Column V data) as in unirradiated hosts (Column I minus Column II data of Experiment A). The same phenomenon can be seen by comparison of the values in Column V with the values in Column I of Experiments B and C. The latter values would presumably be diminished only a negligible amount by subtraction of the corresponding numbers which would remain after antiserum treatment, as judged by the small numbers observed after antiserum with the highest cell dose in each of the experiments. These results can only be explained if colonies produced from cells possessing Friend antigen are represented in the same numbers in the spleens of irradiated and unirradiated hosts injected with equal doses of

### Table 3

<table>
<thead>
<tr>
<th>Suspension injected</th>
<th>Number of mice</th>
<th>Mean number of colonies ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 × 10^6 normal spleen cells</td>
<td>23</td>
<td>7.1 ± 0.6</td>
</tr>
<tr>
<td>1 × 10^6 normal spleen cells incubated with Friend antiserum</td>
<td>13</td>
<td>6.6 ± 1.4</td>
</tr>
</tbody>
</table>

Effect of antiserum on spleen colony-forming activity of normal hemopoietic cells.

* Two groups of 30 (C3H × C57BL)F1 hybrid mice were irradiated at 900 rads before being injected with the suspension of normal spleen cells and the suspension of normal spleen cells incubated with Friend antiserum.

### Table 4

<table>
<thead>
<tr>
<th>Suspension injected</th>
<th>Mean number of lesions per spleen ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^6 infected cells alone</td>
<td>7.0 ± 1.5</td>
</tr>
<tr>
<td>10^6 infected cells incubated with normal serum</td>
<td>7.4 ± 1.9</td>
</tr>
<tr>
<td>10^6 infected cells incubated with Gross serum</td>
<td>7.8 ± 12</td>
</tr>
<tr>
<td>10^6 infected cells incubated with Friend antiserum</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>10^6 infected cells incubated with absorbed Friend antiserum</td>
<td>6.7 ± 1.3</td>
</tr>
<tr>
<td>Absorbed Friend antiserum alone</td>
<td>0</td>
</tr>
</tbody>
</table>

Effects of antisera and normal serum on the production of discrete, macroscopic lesions in the spleens of F1 hybrid mice by Friend virus-infected C3H spleen cells. Infected spleen cells were washed twice in standard medium.

* Mean number of lesions was obtained from the spleens of 7 female (C3H × C57BL)F1 hybrids.
Table 5

| Experiment | No. of spleen cells injected | Unirradiated hosts | Irradiated Hosts (000 rads) | V
|-------------|----------------------------|---------------------|-----------------------------|---
|             |                           | No. of mice | Mean No. of colonies/spleen ± S.E. (control) | No. of mice | Mean No. of colonies/spleen ± S.E. (antiserum-treated)* | No. of mice | Mean No. of colonies/spleen ± S.E. (control) | No. of mice | Mean No. of colonies/spleen ± S.E. (antiserum-treated)* | Difference in mean No. of colonies/spleen ± S.E. between control and antiserum-treated (III — IV) |
| A           | $5 \times 10^6$          | 7          | 1.0 ± 0.2                  | 12          | 2.6 ± 0.5                  | 11          | 1.5 ± 0.3                  | 1.1 ± 0.6                  |
|             | $1.5 \times 10^6$        | 7          | 3.3 ± 0.9                  | 17          | 5.9 ± 0.9                  | 15          | 2.8 ± 0.4                  | 3.3 ± 1.0                  |
|             | $4.5 \times 10^6$        | 7          | 6.0 ± 1.2                  | 13          | 11.3 ± 0.8                 | 15          | 5.5 ± 0.8                  | 5.8 ± 1.0                  |
|             | $1.4 \times 10^7$        | 7          | 13.0 ± 2.7                 | 13          | 11.7 ± 0.4                 | 7           | 17.7 ± 4.3                 |
| B           | $5 \times 10^6$          | 9          | 3.2 ± 0.4                  | 1           | 3.0                       | 1           | 3.0                       | 3.0                      |
|             | $1 \times 10^6$          | 8          | 6.0 ± 0.9                  | 13          | 6.5 ± 0.5                  | 15          | 5.9 ± 0.9                  | 5.9                      |
|             | $2 \times 10^6$          | 8          | 8.9 ± 0.7                  | 9           | 8.8 ± 0.5                  | 7           | 7.0 ± 0.7                  |
|             | $2 \times 10^6$          | 10         | 5.2 ± 0.7                  | 7           | 17.7 ± 4.3                 | 7           | 17.7 ± 4.3                 |
| C           | $4 \times 10^6$          | 10         | 12.0 ± 0.6                 | 11          | 2.8 ± 0.5                  | 24          | 8.1 ± 0.6                  |
|             | $1 \times 10^6$          | 10         | 3.3 ± 1.3                  | 13          | 4.8 ± 0.6                  | 2           | 3.8 ± 0.6                  |
|             | $5 \times 10^6$          | 9          | 1.9 ± 0.4                  | 24          | 8.1 ± 0.6                  |

Effect of Friend antiserum on spleen colony formation by Friend virus-infected C3H spleen cell suspensions in unirradiated and irradiated F1 hybrid hosts.

V Two separate Friend antisera, prepared in the same way, were used in these experiments, one for Experiment A and the other for Experiments B and C. The in vitro Friend virus-neutralizing activity of the first antiserum only was tested; it had a K value of 0.1 (41).

The mean difference was obtained by subtracting from the control value the calculated number of colonies in antiserum-treated hosts at the given cell dose. The calculated colony number in antiserum-treated hosts was derived from the observed value at the highest cell dose.

FV-infected spleen cells. But in the irradiated hosts, colony formation from host cells by FV infection has been critically excluded. Therefore the discrete, macroscopic lesions found in the spleens of unirradiated F1 hybrid hosts after the i.v. injection of FV-infected cell suspensions must also be colonies derived from cells or nearly all of which possess Friend antigen. Moreover, the colonies reach macroscopic size (each is known to contain between $10^6$ and $3 \times 10^6$ cells) as a result of extensive proliferation of these cells in host spleens which are entirely normal. For both reasons, they may be regarded as tumor colonies and their progenitors as tumor cells (see also below).

In irradiated hosts injected with FV-infected cell suspensions, another class of colonies, insensitive to Friend antiserum, evidently exists in significant frequency. We regard these as normal spleen colonies resulting from the proliferation of hemopoietic colony-forming stem cells that are also present in the FV-infected spleen.

Relationship between the Number of Tumor Colonies in the Spleens of F1 Hybrids and the Number of FV-infected C3H Spleen Cells Administered. Serial dilutions of a washed, FV-infected spleen cell suspension were injected i.v. into groups of 8–10 (C3H × C57BL)F1 hybrid mice. Their spleens were scored 9 days later for tumor colonies. The relationship between the mean number of tumor colonies per spleen and the number of FV-infected C3H spleen cells injected was linear (Chart 2). The regression line relating these variables extrapolated back through the origin. Thus it may be concluded...
that each colony seen on the surface of the spleen was probably derived from a single cellular entity in the spleen cell suspension. This entity may be referred to as a TCFU.

**Effect of Time of Sacrifice after Injection of FV-infected C3H Spleen Cells on the Number of Tumor Colonies in the Spleens of F1 Hybrids.** For a colony assay method to be regarded as reliable, it is necessary to show that the number of colonies obtained after the injection of a fixed number of cells remains constant with time, once the colonies have reached a size at which they can be scored. To test the present colony assay method for its reliability in this respect, the following experiment was done. Forty (C3H × C57BL)F1 hybrid mice were injected with $10^6$ washed, FV-infected spleen cells. Groups of 8 mice were sacrificed at 8, 9, 10, 11, and 12 days after injection, and the mean numbers of colonies per spleen were determined. The results (Table 6) show that at 10 days after injection of FV-infected spleen cells, the number of spleen colonies was significantly greater than at 8 days. However, no significant differences in colony number were found from the 9th to the 12th day after injection, while colony size obviously increased. The constancy of colony number with time shows that the method gives a reliable measure of the number of tumor colony-forming units in the FV-infected cell suspensions.

**Distribution of Tumor Colonies in the Spleens of F1 Hybrids.** When various doses of an FV-infected spleen cell suspension were injected into groups of assay mice, the mean number of colonies per spleen was proportional to the number of cells injected. However, the variation in the number of colonies per spleen in each group of assay mice was quite great. To determine the actual distribution of the number of colonies per spleen, 40 F1 hybrid mice were injected with $10^6$ FV-infected spleen cells. Table 7 shows the number of colonies obtained from each of the 40 assay mice. It is apparent, from the fact that the variance was much greater than the mean, that the numbers of colonies in individual spleens after the injection of identical doses of an FV-infected cell suspension into an apparently homogeneous population of mice did not follow a Poisson Distribution.

**Morphology of Tumor Colonies in the Spleens of F1 Hybrids.** Microscopically, colonies in the spleen were found in the red pulp near trabeculae, at or a small distance below the capsule. They were composed of large polygonal cells with faintly basophilic cytoplasm. These cells had clear boundaries which could be seen because the cells were for the most part slightly separated from one another. Each cell contained a large, oval, vesicular, translucent nucleus, one or two large dense nucleoli, and many small, scattered chromatin granules. There were among these cells numerous large mitotic figures and, especially towards the periphery of the nest of large polygonal cells, small round cells. In these, the cytoplasm was difficult to make out, the oval or round nucleus with diameter about half that of the nucleus of the large polygonal cell contained coarse chromatin granules which rendered the nucleus practically opaque. Since small round cells were also numerous in the uninvolved areas of the spleen and since isolated large polygonal cells existed along the irregular periphery, the colonies appeared to merge with the surrounding tissue. Dilated sinusoids filled with blood and containing a few large polygonal cells were frequently seen adjacent to the colonies. Red blood cells could also be seen lying free within and near the colonies. In short, the colonies found in the spleens of F1 hybrid mice injected 9 days earlier with cell suspensions from FV-infected spleens were microscopically indistinguishable from the foci seen in the spleens of susceptible mice after injection with FV. These are depicted in Reference 3.

**Transplantability of Cells from Tumor Colonies in the Spleens of F1 Hybrids.** After injection of FV-infected C3H spleen cell suspensions into (C3H × C57BL)F1 hybrid mice at 10 to 15 days when the colonies could be easily recognized without fixation, individual colonies were removed from the spleens. Cell suspensions were made from 4 of these colonies, the cells were counted ($1.4-9.6 \times 10^6$ cells per colony), and aliquots were injected i.v. into normal (C3H × C57BL)F1 hybrid mice. The cells from all four gave rise to spleen colonies in these hosts. The procedure of removing individual colonies was repeated, and cell suspensions from these again injected i.v. into F1 hybrids. Of the 4 lines started, only 1 gave colonies after the second transfer of $1.6 \times 10^6$ cells. Three sublines were started from this line (cell numbers per colony: $9 \times 10^6-2 \times 10^7$); $\frac{3}{4}$ gave spleen colonies in the third transfer. Each of these also yielded spleen colonies after the fourth transfer. By the fifth transfer, however, no colonies survived from any line.

At the second transfer, aliquots of cells from each of the 3 lines were also exposed to 950 rads of $^{137}$Cs γ-rays and injected into F1 hybrid hosts. Whereas cells from each of the unirradiated colony suspensions gave rise to colonies in these hosts,
those that had been irradiated yielded none. Thus serial transplantation of FV-induced tumor colony-forming cells has been demonstrated through up to 4 transfers in (C3H × C57BL)F1 hybrid mice. The failure to maintain the lines indefinitely could be due either to the number of tumor colony-forming cells injected from each colony being too low or to the possibility that tumor colony-forming cells in normal hosts, like normal hemopoietic colony-forming cells in irradiated hosts, undergo a process of “decline” (37) during serial transplantation.

DISCUSSION

The present work has shown that if cell suspensions from the enlarged spleens of mice recently infected with FV are injected intravenously into normal, genetically compatible adult mice, discrete macroscopically visible lesions develop within a few days in the spleens of these hosts. The experiments described have established that these lesions are colonies which result from the proliferation of cells that have been injected; they are not foci induced by virus, either carried in with the injected cells, or produced by these cells after they have settled in the spleen. The term “tumor colony,” which we have applied to lesions of this type, is justified by the fact that the injected cells, in order to give rise to macroscopic colonies, must undergo extensive proliferation in the spleens of animals which are otherwise in all respects normal; cells from uninfected mice do not lead to colony formation in the spleens of normal hosts. Moreover, cells from these colonies could be serially transplanted for 4 generations in normal F1 hybrid hosts, where they gave rise to macroscopically visible colonies in the spleen.

The linear relation between the mean number of tumor colonies per spleen and the number of nucleated cells injected, with the line extrapolating through the origin (Chart 2), proves that a unit of some kind in the suspensions is responsible for colony formation in the spleens of normal hosts. This unit could be an individual cell, but it could also be a cell aggregate that behaves as a unit in this system. Although its exact nature is still not clear, we may define the TCFU as a cellular entity which reaches the spleen and there gives rise to one tumor colony. With the present method, then, suspensions of FV-infected cells can be assayed quantitatively in terms of their content of TCFU per fixed number of nucleated cells (TCFU ratio).

We have determined the TCFU ratio of 8 FV-infected cell suspensions prepared from the enlarged spleens of animals with early disease and have found values ranging from 0.2 to 2 TCFU per 10⁶ cells. TCFU ratios in the same range were found for individual tumor colonies isolated from host spleens. At least 4 factors could have contributed to these values. One factor is the proportion of injected TCFU’s which settle in the spleen, i.e., the f value (36). Reliable f values were difficult to obtain in this system because even at the highest cell doses, the absolute number of TCFU that could be recovered from host spleens at very early times after injection was low. Preliminary determinations of the f value for TCFU under the present conditions varied from 0.002 to 0.02. A range of f values almost as large as this has been reported for a transplanted lymphoma cell line in AK mice in different experiments (5).

A second factor which could influence the TCFU ratio is the type of host used. (C3H × C57BL)F1 hybrids were chosen as the standard assay animals for the present work because they are relatively resistant to FV and because they would be expected to accept cell transplants from the susceptible C3H parent strain. However, it is now well known that while F1 hybrid hosts do not mount vigorous homograft reactions against parental strain skin or tumor grafts which result in their total destruction, these hosts may nevertheless exert very definite adverse effects on the growth of parental strain cells. A variety of F1 effects have been described. Referred to as “CFU repression” (29), “hybrid resistance” (11), and “allogeneic inhibition,” or “syngeneic preference” (23), these effects have been observed with a number of different cell types, including normal hemopoietic colony-forming cells of bone marrow, spleen, and fetal liver (23, 29) and normal antibody-forming spleen cells (4, 10), as well as with lymphoma (39), carcinoma (22) and sarcoma cells (22, 33). The F1 effects, which are demonstrable in heavily irradiated as well as in normal hosts, can be quite pronounced. McCulloch and Till (29), for example, have observed a 10- to 50-fold reduction in the number of spleen colonies produced by C57BL bone marrow cells in irradiated (C57BL × C3H)F1 hybrids compared to their number in syngeneic C57BL hosts.

The data from the present experiments do not exclude the possibility that F1 effects could be operating in our system. If so, actual TCFU ratios might be higher than those recorded here. Against this is the observation by McCulloch and Till (29) that the reduction in number of spleen colonies produced by normal hemopoietic colony-forming cells from bone marrow does not occur in (C3H × C57BL)F1 hybrid hosts if C3H is the donor parent strain, the strain combination also used in our experiments.

A third factor that could contribute to the TCFU ratio may be the structure of the FV-infected cell population itself. If instead of all cells, only a limited number in the population possessed great proliferative potential, this minority could ensure the continuity of the neoplastic process while the majority of the proliferating cells might be destined to become sterile. Together these two kinds of cells could constitute tumor colonies of macroscopic dimensions, but only the former would be capable of giving rise to spleen colonies when transplanted into other hosts, i.e., only a small fraction of the injected cells might behave as TCFU. A precedent for such a phenomenon exists in the case of the cells of the plasma cell tumor recently studied with the help of the spleen colony method by Bergsagel and Valeriote (in preparation). Only a minor portion of the population was found capable of tumor colony formation in the spleens of compatible hosts. The incapacity of the majority of the cells for sustained proliferation was attributed to either cell death or differentiation to myeloma protein-synthesizing cells. It is interesting in this regard to note that many cells found in spleen foci induced by FV are also differentiated, at least to the extent of being capable of taking up iron (27) and synthesizing heme (unpublished results).

A fourth factor which theoretically could have an influence on the TCFU ratio is an immunologic one. Cells infected with FV are known to possess antigen(s) that are foreign to hosts.
which are otherwise genetically compatible. It is, in fact, the possession of this antigen that conferred the cytotoxic sensitivity which, in the present experiments, permitted a distinction to be made between tumor colony-forming cells and normal hemopoietic colony-forming cells in irradiated hosts. Since the unirradiated (C3H × C57BL)F1 hybrid host would have an intact immune mechanism, the latter might be expected to affect the number of cells capable of forming tumor colonies (compare, for example, Ref. #1). However, this effect can probably be neglected in the present instance, judging from the fact that the number of TCFU in FV-infected spleen cell suspensions was found to be the same whether determined in heavily irradiated or in unirradiated hosts (Table 5).

From the present results, it is evident that at early times after infection, the spleens of FV-infected C3H mice contain at least 2 classes of colony-forming entities. One class is comprised of tumor cells which can produce macroscopically visible colonies by extensive proliferation in the spleens of normal hosts (as well as heavily irradiated ones). All or nearly all of these cells must possess antigen induced by FV infection, for they can be specifically eliminated from the cell population by incubation with Friend antiserum. The other class of colony-forming cells cannot produce colonies in the spleens of normal mice but can do so in heavily irradiated ones; they are insensitive to the cytotoxic action of Friend antiserum. We consider these to be normal hemopoietic colony-forming cells (28, 42).

The presently described method for assaying FV-induced tumor colony-forming cells should provide a useful tool for further investigations on properties of the new cells which appear in the spleens of susceptible mice at early times after infection with Friend leukemia virus.

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A Quantitative Spleen Colony Assay Method for Tumor Cells Induced by Friend Leukemia Virus Infection in Mice

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