A System for Quantitative Studies on Interactions Between Friend Leukemia Virus and Hemopoietic Cells* (33527)

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To understand the role of virus in the development of leukemia in mice, experimental approaches must be directed towards defining and quantitatively measuring cells which are transformed by virus and cells which support its replication. To date, most efforts to this end with mouse embryo cells in tissue culture have only been partially successful (1–3). In addition, such studies may not necessarily render useful information about the interaction of virus and hemopoietic cells *in vivo*.

It is known that if Friend leukemia virus (FV) is administered to lethally irradiated mice, multiplication of virus in the spleen is strongly inhibited (Axelrad and Thomson, to be published) and spleen foci do not develop (4). The spleens of lethally irradiated mice, however, rapidly become repopulated by normal spleen or bone marrow cells injected intravenously (5). Since it is generally agreed that the spleen is the major target organ for FV (6, 7), it was of interest to test the effect of FV in lethally irradiated mice given normal hemopoietic cells.

Materials and Methods. Mice. Sandoz inbred mice (SIM/McK) of both sexes, 5–10 weeks old, were obtained from York Animal Breeders, Richmond Hill, Ontario. Mice were irradiated (900 rads) in the ^{137}Cs unit (8) as previously described (9).

Virus. Friend leukemia virus (FV), originally obtained from the American Type Culture Collection in 1963, and maintained in SIM/McK mice, was used. The virus stocks consisted of resuspended pellets obtained from the high speed centrifugation of infected plasma according to De Harven and Friend (10). Virus preparations were stored in liquid nitrogen.

Virus assays. The spleen forcus assay method was used to determine titers of FV (4). Appropriate dilutions of FV-infected spleen cell suspensions in modified Krebs–Ringer–phosphate medium (9) were subjected to 3 cycles of freezing and thawing and injected intravenously (0.5 ml) into groups of 7–10 mice. Nine days later, the mice were sacrificed and their spleens were fixed in Bouin's fluid. The number of foci on the surface of the spleen was counted with the naked eye. The mean number of spleen foci is linearly related to the titer of FV in the suspension. The titer of FV was expressed as focus-forming units (FFU).

Preparation of cell suspension. Spleen cell suspensions were prepared by mincing the whole spleens and passing the cells with an ice-cold medium through a coarse mesh (110/in.) stainless steel screen (Greening Wire Company, Hamilton, Canada). Aliquotes of cell suspensions were counted in a hemocytometer.

Cell suspensions from other organs except from bone marrow were obtained in the same way as the spleen cell suspensions. Bone marrow cell suspensions were aspirated from the femora of normal mice into an ice-cold medium.

Preparation of Friend antiserum. Groups of SIM mice were injected intraperitoneally with 10^7 formalinized FV-infected spleen cells (11) at weekly intervals for 9 weeks. Cells in the first treatment were emulsified with an equal volume of Freund's adjuvant (containing 0.5 mg/ml of heat-killed dry mycobacteria) prior to injection. Four days after the last injection, the mice were bled and the sera were pooled, heated to 56° for 30 min and stored at —10°. Neutralizing activity of

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the antiserum was tested according to Steeves and Axelrad (11).

Friend virus-induced tumor cell assays. The spleen colony assay method was used to quantitate FV-induced tumor cells (9). Tumor cell suspensions were incubated at 37° for 30 min with or without Friend antiserum. The suspensions were then diluted appropriately and injected into groups of 20 heavily irradiated mice. In addition, the spleen cell suspension which was incubated with Friend antiserum, was injected into a group of 10 unirradiated mice as control. Nine days later, the mice were sacrificed and their spleens were fixed in Bouin's solution. Colonies on the surface of the spleen were counted with the naked eye. Spleen colonies were not observed in normal unirradiatd mice injected with the FV infected spleen cell suspension preincubated with Friend antiserum. This indicated that the concentration of Friend antiserum was high enough to eliminate FV and colony-forming cells bearing Friend antigens (12) in the suspension.

A linear relationship was observed between the number of cells injected from FV-infected mice and the number of tumor colonies produced in the spleens of heavily irradiated hosts (unpublished results). Tumor colonies were considered to be those macroscopic colonies which could be eliminated by incubation of FV-infected spleen cell suspension with specific Friend antiserum from syngeneic mice (9) and which must have developed from cells which possessed antigen(s) induced by FV. Normal SIM hemopoietic colony-forming cells were shown to be unaffected by Friend antiserum. The concentration of FV-induced tumor cells were expressed in terms of tumor colony-forming units TCFU. A TCFU is defined as a cell (or a group of cells acting as a unit) which (i) after intravenous injection reaches the spleen and give rise by proliferation to one tumor colony, and (ii) loses tumor colony-forming ability after reacting with Friend antiserum.

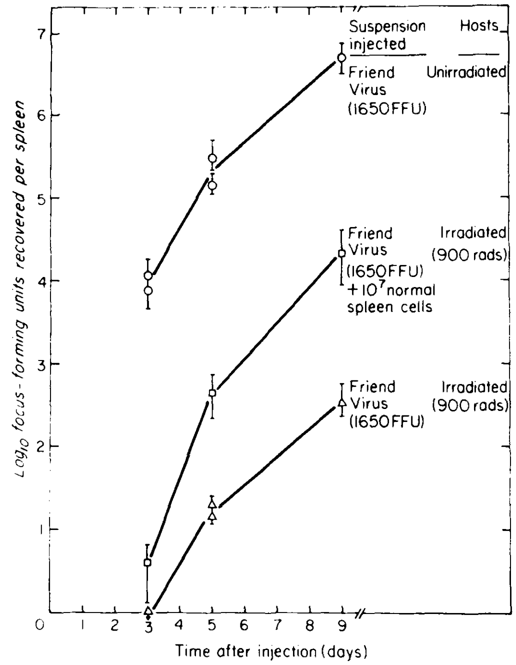

FIG. 1. Recovery of Friend virus from the spleens of unirradiated and heavily irradiated SIM mice with and without addition of normal spleen cells. Mean (of 7) and standard error of FFU recovered per spleen at each time interval are shown.

In vitro *injection of normal cells by Friend virus.* Normal cells and FV suspended in medium were mixed such that 0.5 ml of the suspension contain a desired dose of virus and cells. This suspension was incubated at 37° for 30 min with occasional mixing before being injected into groups of heavily irradiated SIM mice.

Results. The recovery of virus from the spleens of unirradiated and heavily irradiated mice injected with suspenions of FV, is shown in Fig. 1. The amount of virus recovered from the spleens of heavily irradiated mice was, at all times tested, about 10^4-fold less than that from the spleens of unirradiated mice, and it never exceeded the input virus dose. When normal syngeneic spleen cells were added to virus prior to its injection into heavily irradiated mice, the amount of virus was 20–100 times greater than that recovered from irradiated mice injected with FV alone. These results confirm the almost complete prevention of FV multiplication by lethal irradiation of the hosts, and show that virus replication could be restored by the simultaneous administration of cells from normal spleens.

TABLE I. Recovery of Virus from the Spleens of Heavily Irradiated Mice Injected with Suspensions of Normal Spleen Cells and Friend Virus with and without Incubation *in Vitro.*

Suspensions injected into irradiated hosts	Virus (FFU) recovered/host spleen (mean[a] ± SE)
FV (420 FFU)	0
+ 2 × 10^7 normal spleen cells	120 ± 48
+ 2 × 10^7 normal spleen cells (incubated at 37° for 30 min)	1293 ± 375

[a] Mean of 10.

Attempts to increase the yield of FV in the spleens of heavily irradiated mice were successful when FV was incubated with spleen cells at 37° for 30 min prior to injection (Table I). It is known that only a fraction (*f*) of certain hemopoietic cells injected settle in the spleen; e.g., the "*f* value" for colony-forming units is 0.17 (13) while the maximum "*f* value" for focus-forming units of FV is 0.02 (Axelrad and Thomson, to be published). Moreover, the amount of virus recovered from the spleen depends on the dose of virus injected. Thus *in vitro* incubation of cells and virus prior to injection into heavily irradiated hosts allowed virus to travel to the spleen with greater efficiency. Therefore, in later experiments, FV was incubated with cells at 37° for 30 min prior to injection. Whether only adsorption of virus to cells occurs under this condition, or whether infection occurs *in vitro* was not tested in the present experiment.

To ascertain which organs provided cells that gave greater yield of FV in the spleens of heavily irradiated mice, cells from various organs were incubated with FV prior to injection. The results in Table II indicate that virus in increased amounts could be recovered when FV was incubated with spleen or bone marrow cells but not with cells of liver, kidney, lymph node, or thymus before being injected into heavily irradiated mice. This indicated that there were very few target cells for FV replication in those organs. Alternatively, it is conceivable that target cells in those organs did not lodge in the spleens. The amount of virus recovered from the spleens of heavily irradiated mice injected with bone marrow cells and virus was significantly greater than that from mice injected with spleen cells and virus (Table II and III). Furthermore, the amount of virus recovered was greater from the spleens of animals given greater dose of cells previously incubated with FV *in vitro* (Table II). Spleens of heavily irradiated mice injected

TABLE II. Recovery of Virus from the Spleens of Heavily Irradiated Mice Injected with Suspensions of Cells from Various Organs Incubated with Friend Virus *in Vitro.*

Suspensions injected into irradiated hosts[a]		Total no. of cells/host spleen 9 days after injection	Virus (FFU) recovered/host spleen (mean[b] ± SE)
Bone marrow cells (10^7)	+ 500 FFU	1.3 × 10^8	6.3 × 10^4 ± 1.4 × 10^4
Spleen cells (10^7)	+ 500 FFU	6.4 × 10^7	7.7 × 10^3 ± 3.0 × 10^3
Lymph node cells (10^7)	+ 500 FFU	1.6 × 10^7	44 ± 24
Liver cells (10^7)	+ 500 FFU	1.5 × 10^7	26 ± 26
Thymus cells (10^7)	+ 500 FFU	1.5 × 10^7	< 25
Kidney cells (10^7)	+ 500 FFU	1.8 × 10^7	< 25
No cells	+ 500 FFU	1.5 × 10^7	< 25

[a] Suspensions of FV and cells from each group were incubated at 37° for 30 min (4 ml of 2.5 × 10^7 cells/ml + 1 ml of 5000 FFU of FV) prior to injection into 10 heavily irradiated SIM mice.

[b] Mean of 7.

TABLE III. Recovery of Virus from the Spleens of Heavily Irradiated Mice Injected with Spleen or Bone Marrow Cells Incubated with Friend Virus *in Vitro*.

Suspensions injected into irradiated hosts[a]	Total no. of cells/host spleen 9 days after injection	Virus (FFU) recovered/host spleen (mean[b] ± SE)
Virus alone (1000 FFU)	2.1×10^7	81.9 ± 10.7
Spleen cells (10^6) + 1000 FFU	9.5×10^7	$4.7 \times 10^3 \pm 3.0 \times 10^3$
(10^7) + 1000 FFU	1.7×10^8	$1.5 \times 10^4 \pm 1.2 \times 10^4$
Bone marrow cells (10^6) + 1000 FFU	1.2×10^8	$8.9 \times 10^3 \pm 1.8 \times 10^3$
(10^7) + 1000 FFU	1.7×10^8	$7.1 \times 10^4 \pm 1.9 \times 10^4$

[a] Suspensions of Friend virus and cells in each group were incubated at 37° for 30 min (5.4 ml of 1.2×10^8 or 1.2×10^7 spleen or bone marrow cells and 0.6 ml of 1.2×10^4 FFU of FV) prior to injection into heavily irradiated SIM mice.

[b] Mean of 7.

with suspensions of FV and cells from kidney, liver, lymph node, and thymus had about the same number of cells as those from the spleens of irradiated mice injected with FV but no cells, indicating that no repopulation had occurred in those spleens (Table II). In contrast, the spleens of heavily irradiated mice injected with suspensions of FV and spleen or bone marrow cells had 5–10 times more cells than those of irradiated mice injected with FV alone. Some of these cells were shown to be FV-induced tumor cells possessing tumor colony-forming ability (Table IV).

Discussion. It is evident that target cells for FV replication and leukemic transformation were present in both spleen and bone marrow. On a per cell basis, it appears that bone marrow contains more target cells for virus replication than spleen (Table II and III) while they both contain the same number of target cells for leukemic transformation (Table IV). More recent studies on the kinetic of virus and tumor cells indicated that there are more target cells in bone marrow than spleen in *both* virus replication *and* leukemic transformation (Thomson, Steeves and Mirand, to be published). In the case of leukemic transformation, the level of tumor cells in the spleens repopulated with suspensions of *bone marrow* cells and virus reached a plateau before the ninth day postinoculation, while tumor cells in the spleens repopulated with suspensions of *spleen* cells and virus were still growing exponentially 9 days postinoculation. This might account for the same number of tumor colonies observed by the ninth day in these 2 groups (Table IV).

Bone marrow and spleen contain both myelopoietic cells and lymphopoietic cells. However, bone marrow contains *fewer* lymphopoietic cells than spleen. Since cells from bone marrow are *more* efficient in producing virus than spleen cells, it is not likely that lymphopoietic cells are the target cells for FV replication. Further evidence supporting this view is that only small amounts of virus could be recovered from the spleens of irradiated mice injected with suspensions of lymph node or thymus cells and virus (Table II). Thus, the data presented here strongly suggest that the target cells for FV replication and leukemic transformation are myelopoietic cells. Since bone marrow contains more colony-forming cells than spleen (14), it is possible that colony-forming cells are the target cells for virus replication and transformation. The resistance of W^v/W to this virus (15) supports the above suggestion. Whether or not target cells responsible for viral replication are the same as those for leukemic transformation, cannot be decided by the present experiment. While the exact nature of the target cells remains unsettled, there can be little doubt that cells which become virus producers and which are transformed by FV are derived from exogenously administered hemopoietic cells. The environment provided by the heavily irradiated hosts may be necessary for the process of viral replication and

TABLE IV. Recovery of Tumor Cells from the Spleens of Heavily Irradiated Mice Injected with Suspensions of Spleen or Bone Marrow Cells Incubated with Friend Virus *in Vitro*.

Suspensions injected into irradiated hosts[a]	Total no. of cells/host spleen 9 days after injection ($\times 10^8$)	No. of host spleen cells ($\times 10^6$) injected into assay mice	Treatment of assay mice (rads)	No. of colonies/spleen of assay mice (mean ± SE)	Total no. of colony-forming units/host spleen (mean ± SE)	No. of TCFU recovered/host spleen (mean ± SE)
Spleen cells (10^7) + 1000 FFU	1.7	1	900	2.5 ± 0.5	425 ± 85	416 ± 86
		2 + Antiserum[b]	900	0.1 ± 0.1	9 ± 9	
		2 + Antiserum[b]	Unirradiated	0	0	
Bone marrow cells (10^7) + 1000 FFU	1.7	1	900	2.9 ± 0.5	493 ± 85	433 ± 93
		2 + Antiserum[b]	900	0.7 ± 0.4	60 ± 34	
		2 + Antiserum[b]	Unirradiated	0	0	

[a] Suspensions of FV and cells in each group were incubated at 37° for 30 min (5.4 ml of 1.2×10^8 spleen or bone marrow cells and 0.6 ml of 1.2×10^4 FFU of FV) prior to injection into heavily irradiated SIM mice.

[b] Suspensions of infected spleen cells were incubated with Friend antiserum (11) at 37° for 30 min (1 ml of infected spleen cell suspension containing 10^8 cells : 1 ml of undiluted antiserum).

transformation in these cells. This preliminary work suggests that the sequential combination of 2 systems, one operating *in vitro* and the other *in vivo* in heavily irradiated hosts may provide a means of analyzing further the interaction of hemopoietic cells with Friend leukemia virus.

Summary. A system was developed for studying the interactions between Friend leukemia virus (FV) and hemopoietic cells which lead to viral replication and leukemic transformation. The system involves the use of heavily irradiated mice which cannot support the growth of FV in the absence of added hemopoietic cells. When the heavily irradiated mice were injected with the suspensions of virus and normal spleen or bone marrow cells, FV and FV-induced tumor cells were recovered from the spleens of these mice. *In vitro* incubation (37° for 30 min) of cells and virus prior to injection into heavily irradiated mice increased the yield of recoverable virus.

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A Comparative Study of Alkaline Lipolytic Activity in Adipose Tissue of Various Mammals* (33528)

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Human adipose tissue contains a soluble esterase called alkaline lipolytic activity (ALA) (1, 2). This activity can be quantitated by measuring the free fatty acid (FFA) released from tributyrin (pH 8.0) at 47° and qualitatively identified after starch gel electrophoresis by using naphthol esters as substrate and fast blue 2B salt as coupling agent. The latter technique has demonstrated that human adipose tissue ALA exists as five electrophoretically separate isozymes (3). The present paper reports the use of the same techniques to analyze adipose tissue of other mammals.

Methods. Tissue was obtained from several fat depots[1] of nine mammals within 2–3 hr after sacrifice (Table I). The tissue was frozen at —65°[2] until ready for comparison with extracts of human tissue stored in the same manner.

TABLE I. Alkaline Lipolytic Activity (ALA) in Adipose Tissue Extracts.[a]

Mammal	ALA (μeq of FFA/g/hr)
Cat (O)	171.7
Pig (P)	78.1
Rabbit (S)	43.7
Lamb (S)	37.4
Rat (S)	33.9
Human (S)	30.9
Mouse (S)	28.8
Monkey (O)	6.0
Dog (O)	3.0
Steer (S)	0.4

[a] Adipose tissue extracts were prepared as described in the text and activity quantitated by the assay system previously described for measuring ALA in extracts of human adipose tissue. The rise in FFA during incubation was determined and activity expressed as μeq of FFA/g of tissue/hr. Abbreviations for source of tissue: (O) = omental, (P) = pericardial, (S) = subcutaneous.

For study, the tissue was thawed, homogenized in cold 0.15 M KCl in a glass tissue grinder and centrifuged at 25,000g for 10 min after which the aqueous middle layer was recovered and used as the source of enzymatic activity. In order to obtain similar staining after starch gel electrophoresis, the concentration of adipose tissue was varied from 100–1,500 mg per ml of KCl.

Vertical starch gel electrophoresis of the adipose tissue extracts was performed according to the method of Smithies (4). The end trays contained 0.165 M phosphate–citrate buffer (pH 7.0) and a 1:20 dilution of the same buffer was used in preparing the

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[1] Previous studies on human and rabbit adipose tissue indicated that the enzyme pattern on starch gel was the same for various sources of fat.

[2] Although aqueous extracts undergo minor changes in isozyme pattern, even at —65° [Ref. (3)], unextracted tissue can be frozen at —65° without alteration of the isozyme pattern (unpublished data).