Helper Activity of Human Leukemic Tissue Extracts for Leukemia Virus Expression in Mice

(BALB/c mice/*Fv-1^b* gene/Friend virus complex/spleen focus-forming virus)

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ABSTRACT Extracts of cultured human leukemic tissues increased the spleen focus-forming activity of Friend leukemia virus preparations in BALB/c and other partially resistant mice. Such mice carry the *Fv-1^b* gene, which inhibits the expression of helper virus indigenous to the Friend virus complex, and allows co-infecting leukemia viruses of mice, cats, or chickens to substitute for the inhibited helper virus and increase the expression of the spleen focus-forming virus. The helper activity of extracts from human leukemic tissues shared several important properties with that associated with known leukemogenic viruses of animals.

Although the oncogenic RNA viruses (C-type) have not been clearly associated with human leukemia, their role in leukemogenesis in man has been proposed because of their wide distribution among vertebrate species (1), and because of their demonstrated role in leukemogenesis in chickens, mice, and cats (1). In addition, there have been reports of virus particles in leukemic human plasma (2), or in human tumor cell cultures (3, 4), and of RNA-dependent DNA polymerase (analogous to that of RNA tumor viruses) in human leukemic lymphoblasts (5). In the present communication, we provide further evidence to support the hypothesis of viral leukemogenesis in man, based on a new helper assay (6) that appears to be specific for leukemogenic viruses (7).

Recent studies by Pincus *et al.* (8) have demonstrated that the complex *Fv-1* locus in mice controls susceptibility, not just to Friend leukemia virus, but to all laboratory and naturally occurring strains of murine leukemia virus (MuLV). The *b* allele, dominant for resistance to N-tropic MuLVs, reduces the efficiency of spleen focus formation by N-tropic strains of Friend virus. Concurrent work in our laboratory (9, 10) showed that this resistance is probably directed against a lymphatic leukemia-inducing (helper) virus (LLV) in the Friend virus complex, which contributes to the antigenicity (11) and host range (12) of the spleen focus-forming virus (SFFV). From these observations we devised a simple, 9-day assay *in vivo* for viruses that can provide a helper function for SFFV (6). Of many viruses tested to date, helper activity has been restricted to several thylaxoviruses (13), including MuLV isolates (all of 12 tested), a feline leukemia virus (Theilen strain), avian erythroblastosis virus, avian leukosis virus, and

the Bryan high titer and Schmidt-Ruppin strains of Rous sarcoma virus (R. A. Steeves, unpublished data). So far, the only other biological materials that have provided helper activity for SFFV are certain extracts of human leukemic tissues.

MATERIALS AND METHODS

Mice. BALB/c and F₁ (BALB/c × DBA/2) hybrid (CDF₁) mice were obtained from the West Seneca Animal Production Unit of Roswell Park Memorial Institute. Within any experiment the mice were of one sex, and their ages (4-8 weeks) varied by 5 days or less. Because BALB/c mice are B-type (8), *i.e.*, they carry the *Fv-1^b* gene, dominant for resistance to N-tropic MuLVs, both types of mice are phenotypically resistant. This resistance is not absolute, but reduces the infectivity of N-tropic MuLVs in B-type mice to about 10⁻² of that observed in N-type mice (8).

Virus. Friend leukemia virus (14) complex (10) was maintained in random-bred Ha/ICR Swiss (N-type) mice by intravenous passage of splenic cell-free filtrates at 9-day intervals. N-tropic virus stocks were harvested from the supernatant fluid of cell suspensions (20% w/v) of leukemic spleens. These stocks were helper-independent in N-type (Swiss) mice, but helper-dependent in B-type mice (9).

Helper Assay. Helper activity for SFFV has only been observed in B-type mice that are infected with an N-tropic stock of Friend virus complex. Nonspecific enhancement of spleen focus formation has been observed in other susceptible mouse strains (15), but this required application of the enhancing agent 1-2 days before SFFV infection. In contrast, helper activity was most efficient when the agent was administered along with SFFV. The assay, described in detail elsewhere (6), is outlined briefly as follows: The test samples were added to dilution tubes containing ice-cold phosphate-buffered saline (PBS; pH 7.2) and a constant volume of the "indicator" virus stock, Friend-SFFV with its natural N-tropic helper virus. The samples were mixed gently and injected intravenously into BALB/c or CDF₁ mice. Nine days later the mice were killed, their spleens were fixed in Bouin's fluid, and the number of foci (greater than 0.5 mm in diameter) were counted on each spleen. The titer estimate (in focus-forming units (FFU)/ml) of a control sample of SFFV (to which no test sample was added) was subtracted from the titer estimate of SFFV with each sample, and this difference (Δ FFU/ml) was used to express the helper activity of the sample.

Abbreviations: SFFV, spleen focus-forming virus; MuLV, murine leukemia virus; PBS, phosphate-buffered saline; FFU, focus-forming unit; LLV, lymphatic leukemia-inducing (helper) virus; GLV, Gross leukemia virus.

FIG. 1. Spleens from CDF1 mice infected with SFFV *only* (left column) or with the same dose of SFFV added to splenic material from a patient with chronic myelogenous leukemia (right column). The difference between the mean number of foci per spleen in each group ($46.6 - 8.1 = 38.5$), multiplied by the dilution factor (200) of SFFV, gave a Δ FFU/ml value of 7700. In spite of the large difference between the mean focus counts, the mean spleen weights differed by less than 2-fold; this is typical of data obtained only 9 days after infection with Friend virus complex (R. Steeves, unpublished).

Human Tissue Extracts. Spleens and lymph nodes from patients with hematopoietic neoplasms were kindly provided by Dr. A. Mittelman, of the Roswell Park Memorial Institute, while non-neoplastic spleens, removed incidentally during abdominal surgery, were obtained from Dr. J. Fisher, Buffalo General Hospital, and Dr. P. Walczak, Millard Fillmore Hospital. Normal and leukemic blood, bone marrow, and lymph node specimens were provided by Drs. J. Holland, L. Sinks, J. Sokal, and their associates at the Roswell Park Memorial Institute.

Spleens and lymph nodes were washed in PBS, and then minced finely in culture medium (Eagle's medium with 10% heat-inactivated fetal-calf serum). Heparinized blood samples were allowed to stand for about 30 min at room temperature, after which leukocyte-rich plasma was centrifuged at $300 \times g$ (International PR2, 1000 rpm) for 10 min and the cell pellet was suspended in culture medium. Bone marrow was simply expressed directly from a syringe into small bottles of culture medium.

All tissue cells (unless indicated otherwise) were incubated at 37°C in stationary flasks at concentrations of 1×10^6 – 10×10^6 nucleated cells/ml for 2 days. The cells and cell debris were then sedimented from the culture fluids at $9200 \times g$ (Intern-

TABLE 1. Incidence of helper activity in tissue culture extracts of human spleens

Diagnosis	Ratio (no. positive/tested)*		
	Tumor cells in the spleen	Tumor cells undetected in the spleen	Total
Chronic myelogenous leukemia	5/6	0/1	5/7
Chronic lymphatic leukemia	1/2	...	1/2
Lymphosarcoma	0/1	...	0/1
Giant follicular lymphosarcoma	...	1/1	1/1
Reticulum cell sarcoma	0/1	0/1	0/2
Hodgkin's disease	3/5	1/2	4/7
Total	9/15	2/5	11/20

* Extracts were considered positive when the helper activity (in Δ FFU/ml) exceeded 1500. The values varied from 1700 to 8800 Δ FFU/ml for extracts diluted 1/10 in the helper assay after 25- to 100-fold concentration from tissue culture fluids by differential centrifugation. Samples scored as negative for helper activity in this table and 11 samples prepared from normal human spleens gave values of 700 Δ FFU/ml or less.

tional HR-1, 10,000 rpm) for 10 min, after which the supernatant fluids were centrifuged at $75,000 \times g$ (Beckman Rotor #30, 29,500 rpm) for 2 hr. The final pellets, containing subcellular particulate material, were suspended in PBS at 1/25th to 1/100th of the original culture-fluid volume and were stored in sealed ampules at -196°C .

RESULTS

Helper activity of human spleen

Spleens were studied initially because of the relatively large amount of tissue available from each patient. Our results have been encouraging, especially with cases of chronic myelogenous leukemia. The results from one such case are shown in Fig. 1, in which spleens are compared from mice infected with a given dose of SFFV *with* (right column) or *without* (left column) the addition of a leukemic spleen culture extract. Its helper activity was calculated by multiplying the difference in focus formation (Δ foci) between each group by the dilution factor of SFFV to give a value in Δ FFU/ml. To date, this approach has been used to evaluate 20 spleen culture extracts from patients with chronic hematopoietic neoplasms, 15 of which had spread to the spleen. The incidence of significant helper activity (more than 1500 Δ FFU/ml) is shown in Table 1, according to diagnosis and presence or absence of tumor cells in the spleen. The latter criterion has only a slight effect on the results, if one compares the total ratios at the bottom of the table (9/15 versus 2/5). Many more tests will be required to evaluate this and the diagnosis variable more conclusively. Our initial impression at this stage is that chronic myelogenous leukemia is the disease most likely to show helper activity.

Tissue culture extracts were also prepared in the standard fashion from 11 "normal" human spleens and from 7 human embryos (5–13 cm), but all of them failed to provide helper activity over 1000 Δ FFU/ml. The level of 1500 Δ FFU/ml for statistical significance was chosen to allow for variations from one experiment to another in the dilution factor of the indicator and in the standard error of Δ foci.

TABLE 2. Incidence of helper activity in tissue culture extracts of human blood, bone marrow, and lymph nodes

Tissue	Diagnosis	Ratio (no. positive/tested)
Blood	Acute lymphatic leukemia	0/3
	Chronic lymphatic leukemia	1/1*
	Chronic myelogenous leukemia	0/2
	Lymphosarcoma	0/2
	Polycythemia vera	0/2*
Bone marrow	Acute lymphatic leukemia	1/7*
	Acute myelogenous leukemia	0/1
	Chronic lymphatic leukemia	0/1*
	Chronic myelogenous leukemia	0/2
	Lymphosarcoma	0/1
	Reticulum cell sarcoma	0/1
	Polycythemia vera	0/3
Lymph node	Lymphosarcoma	0/1
	Mycosis fungoides	0/1
Total		2/28

* Stationary cultures were maintained for 72–96 hr, instead of the usual 48 hr.

Obvious limitations in the supply of human leukemic spleens prompted us to test extracts prepared from blood, bone marrow, and lymph node cultures. Table 2 shows the number of cultures tested, according to diagnosis, for each tissue. Although the concentration factors were similar to those used to prepare splenic tissue extracts, only 2 out of a total of 28 blood, bone marrow, or lymph node culture extracts have shown significant helper activity to date. Despite this problem, the limited supply of active splenic material has allowed us to make some preliminary observations on variables in culture conditions and on certain characteristics of the helper activity provided.

Culture conditions

Every spleen that became available was suspended (by mechanical mincing) in Eagle's medium with 10% fetal-calf serum and incubated at 37°C as a stationary culture for 48 hr. In addition, some tissue was treated in a different way, such as: trypsinization of spleen fragments to separate the cells, culture of splenic fragments as in a "Maitland" culture, addition of phytohemagglutinin (1 drop/ml), extension of the culture time to 12 days, or immediate harvest of the supernatant fluid without any culture period. As shown in Table 3, these factors consistently reduced the yield of helper activity in extracts that were concentrated in parallel with the corresponding controls.

Characteristics of helper activity associated with human extracts

To examine for similarities or differences between helper activity observed with human extracts and that observed with MuLV preparations, we tested the effects of various procedures on the helper extracts themselves, on the SFFV indicator, and on the assay host. The resultant helper activities (Table 4) were expressed in Δ foci instead of the customary Δ FFU/ml, because of the large SFFV dilution factors required for the last two procedures. Exposure of a human extract to ultraviolet (UV) light eliminated its associated helper activity, as did injection of the extract 24 hr before injection of the

TABLE 3. Influence of various culture conditions on helper activity of human splenic extracts

Procedure	Helper activity expressed as mean Δ FFU/ml $\pm$ standard error*	
	Test	Control†
Trypsinization of spleen cells	1,440 $\pm$ 520 (15.3–8.1)*	7,700 $\pm$ 1,300 (46.6–8.1)
Culture of splenic fragments ("Maitland" culture)	1,300 $\pm$ 370 (7.0–0.5)	2,000 $\pm$ 290 (10.5–0.5)
Use of phytohemagglutinin	500 $\pm$ 120 (3.4–1.5)	6,100 $\pm$ 1,000 (24.5–1.5)
Culture for 14 days	2,700 $\pm$ 600 (8.0–1.3)	8,400 $\pm$ 1,300 (24.0–3.0)
No culture period	1,600 $\pm$ 670 (7.7–1.5)	6,100 $\pm$ 1,000 (24.5–1.5)

* In parentheses are the mean numbers of foci per spleen that were used to calculate the helper activity in Δ FFU/ml. For example, in the test on trypsinization, 15.3 is the mean number of foci in mice injected with SFFV and human extract, and 8.1 is the mean number of foci in mice injected with SFFV only. The difference (Δ foci), multiplied by the dilution factor for SFFV, is the helper activity in Δ FFU/ml.

† The various control cultures were maintained as stationary cultures of single cells in Eagle's medium with 10% fetal-calf serum for 48 hr. No trypsin or phytohemagglutinin was used.

SFFV indicator. As might be expected from the latter observation, human extracts by themselves did not demonstrate spleen focus-forming activity. We also tested whether alterations in the antigenicity or host range of the indicator virus would affect helper activity. Preliminary results had led us to

TABLE 4. Characteristics of helper activity associated with human leukemic tissue extracts

Procedure	Helper activity expressed as mean Δ foci $\pm$ standard error*	
	Test	Control†
Extract UV-irradiated (1.6×10^2 ergs/mm ²)‡	3.1 $\pm$ 2.6 (11.5–8.4)*	10.0 $\pm$ 3.1 (18.4–8.4)
Extract injected 24 hr before SFFV(LLV)	0.3 $\pm$ 2.6 (5.7–5.4)	10.1 $\pm$ 3.9 (15.5–5.4)
Indicator substituted by SFFV(GLV)§	1.3 $\pm$ 0.7 (2.1–0.8)	10.1 $\pm$ 3.9 (15.5–5.4)
Indicator substituted by NB-tropic¶ SFFV(LLV)	1.3 $\pm$ 1.2 (5.4–4.1)	9.0 $\pm$ 2.1 (10.8–1.8)
Host substituted by N-type¶ DBA/2 strain	1.5 $\pm$ 1.7 (4.0–2.5)	9.0 $\pm$ 2.1 (10.8–1.8)

* Numbers in parentheses are the mean numbers of foci used to calculate the helper activity in Δ foci. (See footnote to Table 3.)

† Controls were tested with untreated human extract, combined with N-tropic¶ SFFV(LLV) indicator, and injected into B-type¶ mice.

‡ Exposed to ultraviolet (UV) light at 1380 ergs/mm² sec⁻¹ (at the surface of the extract, 10 cm from the source) for 20 min.

§ Symbol for the Gross leukemia virus pseudotype of SFFV, prepared according to the method described in ref. 11.

¶ See ref. 8 and text for definitions of these terms.

FIG. 2. Linear relationship between helper activity (mean $\pm$ SE) and the dilution factor of a tissue-culture extract prepared from the spleen of a patient with giant follicular lymphosarcoma. At the $1/10$ dilution the helper activity was more than 6000 Δ FFU/ml. The Y-intercept represents the theoretical helper activity of undiluted helper, and indicates a value of 5×10^4 Helper Units (HU)/ml (6).

believe that the Gross leukemia virus pseudotype (12), SFFV(GLV), might function as a more sensitive indicator, but follow-up experiments did not confirm this. In fact, SFFV-(GLV) was generally a poorer indicator of helper activity than the original N-tropic SFFV(LLV), and in the results given here showed no helper activity at all. Use of BALB/c-adapted (NB-tropic) indicator eliminated helper activity altogether, probably because the indigenous LLV was not inhibited in the B-type assay mice. Similarly, helper activity was abolished when N-type mice were used to quantitate N-tropic indicator virus. Dilution of a human extract resulted in helper activity that was inversely proportional (on a log/log plot) to the dilution factor of the human extract (Fig. 2). All of these characteristics of human extract-associated helper activity have been also observed with MuLV-associated helper activity (6).

DISCUSSION

The helper activity associated with human leukemic tissue extracts showed many similarities to that observed in association with MuLV preparations. It was readily sedimented from tissue culture supernatant fluids and sensitive to UV light, and it required injection along with an N-tropic SFFV(LLV) indicator into a B-type host. Furthermore, the similarity was made even more plausible by the apparent specificity of the helper assay method for leukemia viruses of mice, cats, and chickens (7).

We are fully cognizant, however, that some less-specific mechanism could have caused the present results, for certain parallels with MuLVs are lacking. For example, preliminary electron microscopic examination has not revealed obvious C-type or other virus-like particles in our human extracts. We have not been able to demonstrate, with human extracts, rapid growth of a helper-inducing entity in mice, or conversion of the antigenicity of SFFV by mixed "infection" of suckling B-type mice, as we have with MuLVs (10, 11). Finally,

MuLV-infected spleen cells differ from the human leukemic spleen cells, since the former provide more helper activity if left uncultured. These differences are not altogether surprising, however, and we remain impressed by the compelling similarities referred to above.

The possibility that a foreign antigen or bacterial endotoxin could have enhanced spleen focus-formation by stimulating target cells for SFFV is unlikely, because such a mechanism: (a) would require exposure to the antigen or endotoxin at least 1 day before SFFV infection (15) rather than at the same time, (b) would not be expected to give the distribution of low activity observed in Tables 1-3, and (c) would not require interaction with an N-tropic SFFV indicator in B-type mice (Table 4). Similar reasoning distinguishes the helper activity described here from "accelerant" (16) or "XYZ" (17) factors that have been shown to enhance the malignant process associated with transplantation of experimental tumors. It is also noteworthy that Mycoplasma (pleuropneumonia-like organisms) that are known to exist more frequently in tissues of immunodepressed leukemic patients (18), failed to provide helper activity for SFFV.

This preliminary study has encouraged us to continue with further investigations in this area. Current studies are directed toward: (A) expanding the survey of human splenic extracts for helper activity, (B) further testing of nonsplenic human extracts, and (C) characterizing the properties of the human helper-associated agent with virologic, serologic, and electron microscopic techniques.

NOTE ADDED IN PROOF

Three recent experiments (controlled more carefully than earlier ones to avoid excessive cell damage) have revealed that immediate centrifugation of leukemic-cell suspensions without any culture period provided as much or more helper activity than suspensions incubated for 48 hr. This lack of a culture requirement is similar to unpublished results obtained with LLV-infected mouse spleens.

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