Properties of a Lymphocytic Leukemia Agent Isolated From Ha/ICR Swiss Mice.* (29230)

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An agent which induces lymphocytic leukemia in a large percentage of Swiss mice was isolated during experiments which originated with the assay of human tumors and leukemias for the presence of oncogenic agents(1).

In October, 1958, 15 newborn Hauschka ICR (Ha/ICR) Swiss mice were injected with a cell-free filtrate of the bone marrow from a young woman with acute myelocytic leukemia. Four and one-half months later, one of these mice, a female, developed a mammary adenocarcinoma, with lymphoid hyperplasia in the adjacent lymph nodes. Passage of kidney, liver and spleen homogenate was made from this animal; subsequently, blind passages of similar homogenates were made to suckling mice. Parallel passages of normal organs from normal mice of comparable ages served as passage controls throughout.

One female from the fifth passage developed leukemia at 7 months of age. A cell-free filtrate of kidney, liver and spleen was concentrated (5 × the original 10% suspension of tissue, weight/volume) and injected into 9 newborn Swiss mice. Three females from this litter developed leukemia 8½-10½ months later. Two successive passages were then made. The supernatant fluid from a 10% suspension of leukemic tissue was injected into suckling (9 days old) Swiss mice, followed by another blind passage to 11-day-old mice one week later.

Seven months later the 8th passage† was made from 2 female mice, one with lymphocytic leukemia and mammary carcinoma, the other with mammary carcinoma. The present studies originated from this passage. A cell-free filtrate of tissues from 2 mice which developed leukemia at 5 and 6 months of age respectively yielded a high percentage of leukemias with a shortened latency. The agent is now in its 14th passage.

Materials and methods. Animals. Pregnant Hauschka ICR (Ha/ICR) Swiss mice were obtained from Dr. Theodore Hauschka's breeding colony at Roswell Park Memorial Institute. Rf strain mice were random-bred from a nucleus obtained 2 years ago from Roscoe B. Jackson Memorial Laboratory. Pregnant Sprague-Dawley rats were purchased from the Charles River Breeding Laboratories. All animals received Rockland diet and tap water *ad libitum*. Intercurrent infection was treated by addition of Achromycin (Lederle) to the drinking water (50 mg/500 ml) for 1-2 weeks. All animals were inspected weekly, and sacrificed *in extremis* whenever possible. Sections were taken at random from the various groups of animals, and whenever necessary to establish the diagnosis of leukemia or other cause of death.

Cell-free filtrates were made routinely from the kidney, liver, spleen and lymphoid tissues (thymic lymphoma and lymph nodes) of leukemic mice. Tissues were homogenized in sterile Ringer's solution containing 1% penicillin-streptomycin. The final dilution was a 10% weight/volume suspension of the tissue. The homogenate was clarified for one hour at 5000 rpm in a model L Spinco centrifuge (#40 rotor) at 4°C and the supernatant fluid was then passed through 0.45 μ millipore filters, using *E. coli* markers. Post-filtration sterility was also checked in Sabouraud's and thioglycollate broth. Newborn mice received 0.1 ml of filtrate subcutaneously in the shoulder region; weanling mice received 0.2 ml subcutaneously in the back.

Antiserum against the agent was produced in rabbits by injection of a standard 10%

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† This was designated, erroneously, as the 9th passage in the abstract published in *Proc. Am. Assn. for Cancer Research,* April 1963.

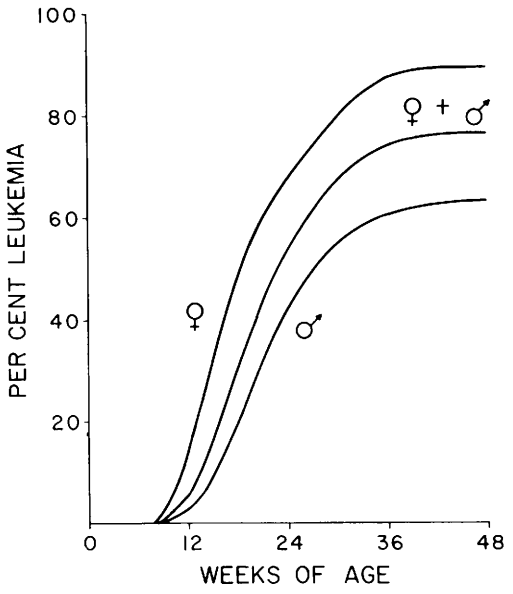

FIG. 1. Cumulative incidence of leukemia in Ha/ICR Swiss mice injected at birth with cell-free filtrate.

cell-free filtrate in each of the 4 foot pads and subcutaneously on each side of the back. A 1:1 mixture of filtrate and Freund's complete adjuvant was injected initially, followed 3 weeks later by a booster injection of filtrate alone without adjuvant. One week later a second booster injection (subcutaneously in the back only) was made, and serum collected after 7 to 10 days. Each rabbit received a total of 7.5 ml of filtrate. Infectivity of the filtrate was assayed in newborn Swiss mice. Antiserum to normal mouse tissue (kidney, liver and spleen) was prepared in a similar manner. Pre-immunization serum (normal rabbit serum) was obtained from each rabbit. All sera were inactivated at 56°C for 30 minutes before use. The antiserum was tested for neutralizing activity by incubation with an equal volume of leukemic filtrate (1:1) at room temperature for 20-30 minutes and inoculation into newborn mice. Mice receiving filtrate incubated with antiserum were given twice the volume of inoculum.

Ether treatment of filtrate. Nine volumes of tissue filtrate were thoroughly mixed with one volume of diethyl ether (reagent grade) and allowed to stand overnight in the refrigerator. Nitrogen gas was then bubbled through the mixture for 10-12 minutes to remove the ether, and the filtrate allowed to stand in open Petri dishes (covered with filter paper) at room temperature until all trace (odor) of ether had disappeared (30-90 minutes).

Results. Biological properties. The incidence and latency of leukemia induced in Ha/ICR Swiss mice by injection of cell-free filtrate at birth is shown in Fig. 1. These curves represent the combined results from the 9th, 10th and 11th passages of this agent. The incidence and latency vary somewhat in different passages, but the greatest number of leukemias occur between 3 and 6 months of age. Incidence is higher and latency shorter in females.

Leukemia occurs spontaneously in less than 1% of mice of this strain. Of 837 control mice observed for a period of 12-14 months, only 5 (0.6%) developed leukemia, one each at 7, 8 and 10 months, and 2 at 12 months of age. Four of the 5 mice were females. About one-half of these controls had received tissue culture fluids or other diluents at birth, and the rest were uninoculated; they were maintained under conditions identical with those of experimental animals. These data are comparable to those previously reported for breeding colonies of this strain at Roswell Park Memorial Institute(1).

Grossly, advanced leukemia involved all lymphoid organs with infiltration of other organs (Fig. 2). Involvement of the thymus

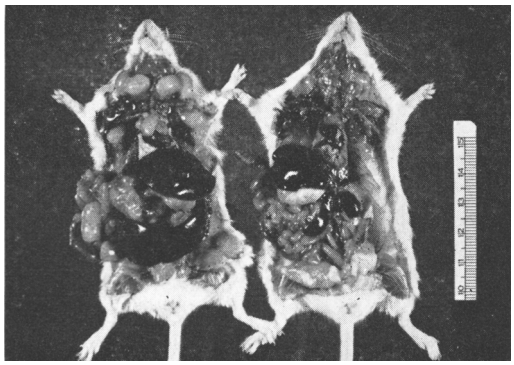

FIG. 2. Advanced leukemia in a 164-day-old Swiss mouse which had been injected at birth with cell-free filtrate, showing extensive involvement of lymph nodes, spleen and liver, and a small thymic lymphoma, more pronounced in the right lobe. Normal animal, on right, is same age and sex.

varied, ranging all the way from huge lymphomas with or without much involvement of other lymphoid organs to atrophic thymuses with extensive involvement of other organs. This was not a time-related phenomenon, because many of the early leukemias showed extensive generalization without grossly observable involvement of the thymus. Systematic microscopic study of all thymuses of infected mice was not done.

Microscopically, the characteristic leukemic cell was the medium to large lymphocyte. The normal architecture of the spleen and lymph nodes was usually completely obliterated. Kidney and liver very frequently showed massive infiltration, and salivary glands, pancreas, ovary and uterus, and subcutaneous tissues were frequently invaded by leukemic cells. The bone marrow was usually involved.

The animals usually died within one to 2 weeks after leukemia became grossly apparent. At this time the peripheral blood abnormalities consisted of elevated total leucocyte counts, anemia and an increased number of immature cells of the lymphoid series.

Weanling Ha/ICR mice were relatively resistant to leukemia induction by this agent. Of 435 mice injected at 3 to 4 weeks of age with cell-free filtrate, only 36 (8%) developed leukemia in 26-47 weeks. Of 438 mice injected at birth with the same filtrates, 293 (67%) developed leukemia in 11-43 weeks. These data were compiled from several experiments which ran concurrently.

Rf strain mice were found to be just as susceptible to this agent as Ha/ICR mice. A total of 70 newborn Rf mice were injected with 5 different filtrates; 56 mice (80%) developed leukemia in 13-40 weeks. Forty of 49 Ha/ICR mice (82%) injected at birth with 3 of the 5 filtrates developed leukemia at 13-33 weeks. No leukemias occurred in 117 uninoculated control Rf mice during this period. The leukemias obtained in Rf mice were of the lymphocytic type, similar in all respects to the leukemias induced in Ha/ICR mice.

A cell-free filtrate from an Rf leukemia (110 days after injection) was injected into newborn Rf and Ha/ICR mice, and induced leukemias in 82% of Rf and 77% of Ha/ICR mice at 17-27 weeks and 12-36 weeks respectively. One Rf leukemia was carried by subcutaneous cell graft through 7 transplant generations in adult Rf mice. The incidence of successful cell grafts was 54-100% over a period of 16-71 days. The failure to obtain 100% successful "takes" was thought to be due to some histoincompatibility as the result of random breeding over the past 2 years. Cell-free filtrate from the second transplant generation yielded leukemias in Rf and Ha/ICR mice injected at birth (60% and 43% respectively in 10-28 weeks). Attempts to recover virus from one of the latest transplant generations are in progress.

Fifty-nine newborn Sprague-Dawley rats were injected with cell-free filtrate; 10 rats (17%) developed leukemia of the lymphocytic type. The first leukemia occurred at 15 weeks; the others occurred at 20, 22, 29, 32, 39 and 46 weeks of age. No leukemias occurred in 16 control Sprague-Dawley rats observed over a comparable period of 58 weeks, nor in approximately 100 rats which received other materials and were observed for at least one year. A cell-free filtrate prepared from leukemic tissues from the first rat to develop leukemia (15 weeks) was injected into 47 newborn Ha/ICR mice. Leukemia of the characteristic lymphocytic type occurred in 36 mice (77%) at 16-44 weeks of age.

Physical properties. Nearly maximal infectivity of the agent was retained after storage at refrigerator temperature (4°C) for 48 hours (Fig. 3). There was some loss of infectivity after storage at room temperature for 24 hours. The latency was extended in both cases. Freezing reduced infectivity somewhat (Fig. 4), with the greatest loss occurring after storage at —20°C for 1 month. Another cell-free filtrate, stored for 6 months in the CO_2 deep freeze, yielded only 29% leukemias in Ha/ICR mice in 16-36 weeks, as compared to 78% in 11-36 weeks with fresh filtrate. The remaining mice are still under observation. In an earlier experiment, the agent also survived 2 freeze-thaw cycles (40% leukemias).

Infectivity was completely abolished by

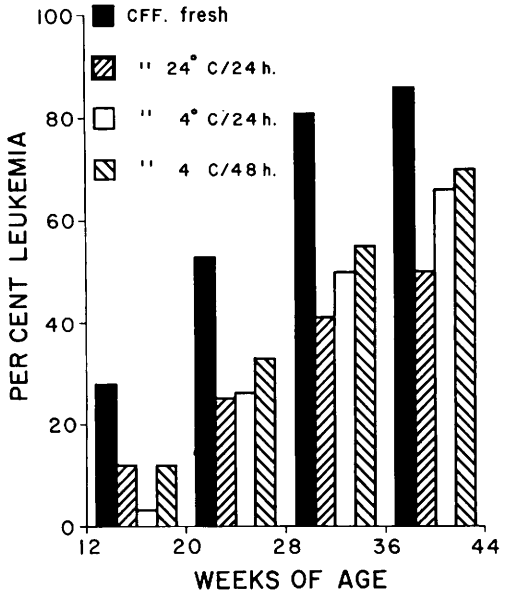

FIG. 3. Stability of leukemia agent at room and refrigerator temperature for 24-48 hr. Cumulative incidence of leukemia in Ha/ICR Swiss mice injected at birth with cell-free filtrate (CFF). Same filtrate throughout.

heating at 56°C for 30 minutes (Table I). However, treatment of the filtrate overnight with ether, at refrigerator temperature, did *not* inactivate it.

Incubation of filtrate with specific antiserum produced in rabbits significantly lowered the incidence of leukemia and extended the latency by 5-8 weeks (Table II).

Discussion. Although this agent was isolated in the course of experiments which originated with human leukemia, it is doubtful that it is directly related to the original human material. It is probably a leukemia virus of murine origin which was somehow activated during our experiments and whose virulence was increased by subsequent selective passages.

Grossly and microscopically, the lymphocytic leukemia induced by our agent is strikingly similar to those induced by the Gross and Moloney viruses(2), and it is tempting to speculate that one of these agents may have been activated in our mice. However, there are some differences between the biological and physical properties of our agent and the Gross and Moloney viruses(2,3,4).

TABLE I. Inactivation of Infectivity of Leukemia Agent in Ha/ICR Swiss Mice Injected at Birth.

Material inj	No. mice*	No. leukemias	% leukemia	Time interval (wk)
CFF	25	13	52	12–40
" 56°C/30 min	37	0		
" 65°C/30 min	34	0		
" 80-85°C/30 min	31	0		
CFF	21	19	90	14–29
" 4°C/overnight	40	31	78	14–35
" Ether 4°C/overnight	33	28	85	12–37

CFF = cell-free filtrate.
* Surviving at 56 days of age.
Experiments terminated at 43 wk.

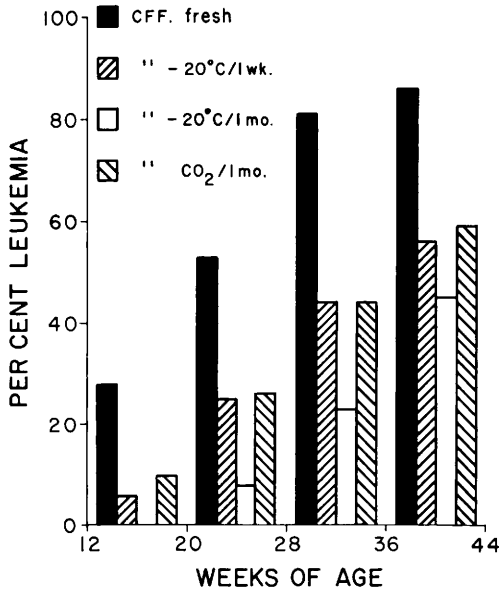

FIG. 4. Effect of freezing on stability of leukemia agent. Cumulative incidence of leukemia in Ha/ICR Swiss mice injected at birth with cell-free filtrate (CFF). Same filtrate as in Fig. 3.

The infectivity of our agent is low for weanling mice, and in this respect differs from the Moloney virus, the infectivity of which is high for weanling and adult mice. However, this could be related largely to virus titer and host response. Our agent is leukemogenic for newborn Sprague-Dawley rats and for more than one strain of mice (other strains are currently under investigation), as are the Gross and Moloney agents.

TABLE II. Neutralization of Infectivity of Leukemia Agent in Ha/ICR Swiss Mice by Rabbit Antiserum.

Exp group	No. mice*	No. leukemias	% leukemia	Time interval (wk)
V–	27	25	93	9–37
V–VRAS	27	4	15	17–25
V–NRAS	30	23	77	12–32
V–NRS	30	24	80	11–36
–VRAS	38	0		
–NRAS	30	0		
–NRS	39	0		
Uninoculated controls	40	0		

V = leukemia virus, CFF. –VRAS = leukemia virus rabbit antiserum. –NRAS = normal mouse tissue rabbit antiserum. –NRS = normal rabbit serum.

The cell-free filtrate used for immunization of the rabbits yielded 74% leukemias in Swiss mice in 15-34 wk after injection at birth.

* Injected at birth and surviving at 56 days of age.

Experiment terminated at 40 wk.

Heating at 56°C for 30 minutes inactivates our agent, but freezing results in only slight loss of infectivity. In this respect, it is similar to both the Gross and Moloney viruses, as well as to several other murine leukemia viruses. However, our agent retains its infectivity after treatment overnight with ether, as does the Moloney virus; the Gross virus, on the other hand, is destroyed by ether.

The variable involvement of the thymus in the leukemias induced by our agent may be largely a host response. Previous experience with spontaneous Ak leukemia and Gross virus passage A induced leukemia in Ak/Z mice(5) indicated that thymic lymphomas were the predominant feature in early leukemias, and that generalized leukemia without thymic involvement usually occurred in the late disease. In the experiments reported here, many of the leukemias were generalized with extensive involvement of the lymph nodes, spleen, and other organs and with little, if any, gross involvement of the thymus; this occurred very frequently in the early disease. It is quite possible that leukemic changes occurred in the thymuses of injected mice in the preleukemic period, followed by seeding of other lymphoid organs (spleen and lymph nodes) with leukemic cells. Then, later, the leukemic process in the thymus may have been retarded or inhibited, resulting in small lymphomas or actual atrophy. A pathogenetic study should clarify this point.

Serological comparison of this agent with other murine leukemia agents is in progress. Growth curve studies, now under way, indicate that virus can be recovered from tissues of mice as early as 2 weeks after injection at birth with cell-free filtrate. The interval after virus infection when viral concentration is greatest remains to be determined.

Summary. An agent has been isolated from Ha/ICR Swiss mice which induces lymphocytic leukemia in approximately 80% of Swiss mice injected at birth with cell-free filtrates. This agent retains its infectivity after storage at room temperature for 24 hours, at 4°C for 48 hours, at —20°C for 1 month, and in the CO_2 deep freeze for 1 month. There is some loss of infectivity under all storage conditions, greatest at —20°C for 1 month. Storage in the CO_2 deep freeze for 6 months reduces infectivity to about one-third. In all instances, latency is extended. The agent is inactivated at 56°C for 30 minutes, but is not destroyed by exposure to ether overnight. It can be neutralized by incubation with specific antiserum produced in rabbits. Sprague-Dawley rats and Rf strain mice are susceptible to infection when injected at birth; weanling Swiss mice are resistant to infection. Similarities and differences between this agent and other murine leukemia viruses are discussed.

1. Grace, J. T., Jr., Mirand, E. A., Millian, S. J., Metzgar, R. S., *Fed. Proc.*, 1962, v21, 32.

2. Moloney, J. B., *ibid.*, 1962, v21, 19.

3. Sinkovics, J. G., *Ann. Rev. Microbiol.*, 1962, v16, 75.

4. Stansly, P. G., *Progr. exp. Tumor Res.*, 1963, v3, 216.

5. Levinthal, J. D., Buffett, R. F., Furth, J., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 455.

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