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Development and Serial Cell-Free Passage of a Highly Potent Strain of Mouse Leukemia Virus.* (23080)

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After it was found that filtered extracts prepared from tissues of leukemic Ak or C58 mice may induce leukemia following inoculation into newborn mice of a susceptible strain (1-4), it became apparent that the results of individual experiments may vary to a considerable degree, depending on the particular extract used. Of 70 filtrates tested, each prepared from a different donor, 18 were found completely inactive(5). Similar observations were made by Woolley(6). Furthermore, extracts prepared from C3H mice in which leukemia had been induced with cell-free Ak leukemic agent, proved more active than those prepared from Ak mice with spontaneous leukemia(4-5). It appeared reasonable to assume, therefore, that selecting a particularly active extract and passing the agent through several successive newborn hosts, may eventually result in a strain of leukemic virus of high potency(5,7). To find a potent extract, groups of litters of newborn C3H mice were inoculated with cell-free leukemic extracts prepared from different donors. Among inoculated mice, those developing leukemia at earliest date were then used as donors for preparation of new cell-free extracts, which were inoculated into newborn mice. The first mice developing leukemia were used as donors for preparation of new cell-free extracts, which were inoculated into newborn mice, etc. A number of different passage-strains of leukemic agents were thus obtained. Of the leukemic passage-strains developed during these experiments, the one here reported and designated "passage A" proved to be highly pathogenic. Filtered extracts prepared from the last few passages of this agent induced acute, generalized, lymphatic leukemia in most of the inoculated mice after only 3½ months. Furthermore, extracts containing the passage virus could be preserved by lyophilization, or simply by freezing in carbon dioxide ice at −70°C in sealed ampoules without apparent loss of activity.

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Methods. Preparation of leukemic extracts.

From mice with advanced leukemia, parts of liver, spleen, mediastinal and mesenteric tumors, and peripheral lymph nodes, were removed aseptically, weighed, ground in mortar with sterile physiological saline added to obtain 20% concentration, centrifuged at 0°C, first at 3000 rpm (1400 x g), for 15 minutes, then at 9500 rpm (7000 x g), for 5 to 10 minutes; the final supernate was used for inoculation. In experiments in which a filtrate was to be inoculated, approximately 10 ml of the 9,500 rpm supernate was mixed with 0.5 ml of 1:2000 dilution of fresh broth culture of *E. coli*, passed through Selas, porosity 02 or 03, porcelain filter candle, and the filtrate then inoculated into newborn mice (in each instance the filter candle retained *E. coli*). In a few experiments the 9,500 rpm supernate, or the filtrate, were centrifuged in Spinco L ultracentrifuge at 40,000 rpm (144,000 x g) for a few minutes to 3 hours, to concentrate the virus; the resuspended sediment pellet was then used for inoculation. In one experiment (fourth passage), blood plasma of the leukemic donor was used for preparation of the extract. All extracts were kept at 0°C, and injected within 48 hours. *Test animals.* Newborn C3H, or foster nursed C3H(f) mice, both of Bittner substrain(4), were used for inoculations. Most animals were <12 hours, many <6 hours, none >16 hours old, at time of inoculation. Those mice that died when less than 2 months old were not included in the tabulation; this concerns particularly the mortality, usually, however, not exceeding 25%, occurring among infant mice within hours or days after inoculation.

Results. Serial, cell-free passage of the leukemic agent from host to host. Only a cell-free passage, consisting of successful transmission of the leukemic agent from host to host, inoculating either a centrifuged or filtered extract, was considered a consecutive "passage," and given a passage number. In one instance, when after the fifth passage newborn mice were not immediately available for inoculation, yet the donor had to be sacrificed, leukemia was carried for 3 successive transplantations by intraperitoneal cell-grafts into adult hosts of the same C3H inbred line; such cell-transfer, however, was considered to be within the same passage, and was designated by the same passage number, *i.e.* "passage 5-a," "5-b" and "5-c." Only succeeding cell-free transfer was then designated "6th passage." This procedure was adopted because it appeared questionable whether cell-transfer would increase, or even sustain, the infective potency of the leukemic agent. Transplantations were carried out at a time when sufficient information was not yet available to suggest that the leukemic agent could be preserved by freezing at −70°C.

Origin of the leukemic virus strain. A breeding female mouse No. 557 of the Ak-n strain of our colony, which developed spontaneously lymphatic leukemia at 7½ months of age, was sacrificed; liver, spleen, mesenteric and mediastinal tumors, removed aseptically, were ground, with physiological saline solution added to make a cell suspension of 20% concentration. This cell suspension was then centrifuged at 3,000 rpm for 15 minutes, and the supernate was dry-frozen.

First passage. The lyophilized extract was preserved in a sealed ampoule in refrigerator at 4°C for 5½ months. The content of the ampoule was then resuspended in 2 ml of sterile physiological saline, and inoculated into a newborn C3H litter consisting of 3 females and 4 males. All 3 females developed generalized leukemia at 4½ to 5½ months of age; of the 4 inoculated males, 3 developed leukemia at 4½, 5½ and 10½ months of age, and 1 died at 10½ months with no evidence of tumors or leukemia.

Second passage. One of the leukemic females from the preceding experiment served as donor for preparation of an extract. The leukemic cell suspension was centrifuged first at 3,000 rpm for 15 minutes, then at 9,500 rpm for 10 minutes. The second supernate was centrifuged in Spinco Model L Ultracentrifuge (rotor No. 40) at 40,000 rpm (144,000 x g) for 90 minutes. The sediment pellet from final centrifugation resuspended in 2 ml of sterile physiological saline, was inoculated into a newborn C3H(f) litter consisting of 2 females and 1 male. All 3 inoculated mice de-

veloped generalized leukemia after 1½ to 3½ months.

Third passage. One of the leukemic females from the preceding passage was used as donor. The leukemic cell suspension was first centrifuged at 3,000 rpm for 15 minutes, then at 9,500 rpm for 10 minutes. The second supernate was then centrifuged in Spinco Ultracentrifuge for 3 hours at 40,000 rpm (144,000 x g); the sediment pellet from final centrifugation was resuspended in 2 ml of physiological saline solution and inoculated into 5 newborn C3H males: 2 developed leukemia at 6 and 7½ months of age respectively; of the remaining 3 mice, 1 died when 4 months old and 2 at 17 months without signs of either tumors or leukemia.

Fourth passage. From a leukemic male from the preceding passage, in ether anesthesia, 0.5 ml of blood was removed directly from heart and immediately diluted with 7 ml of physiological saline solution containing a few drops of heparin (the 1% stock solution of Upjohn heparin was prepared without phenol preservative). The diluted blood was centrifuged at 3,000 rpm for 10 minutes, and the supernate then centrifuged at 9,500 rpm for 10 minutes. The second supernate, designated "plasma," was inoculated into 7 newborn C3H mice; of these, 3 developed leukemia at 5 to 6 months of age, 3 other mice developed bilateral parotid gland carcinomas at 4 to 7½ months of age, and 1 died without signs of either tumors or leukemia at 6½ months of age.

Fifth passage. One of the leukemic mice from the preceding passage was used as donor. A leukemic cell suspension prepared from mediastinal tumor, liver, spleen, and peripheral glands, was centrifuged at 3,000 rpm for 15 minutes and then at 9,500 rpm for 5 minutes. The final supernate was inoculated into 5 newborn C3H mice. All developed generalized leukemia at 3 to 7 months of age.

Sixth passage. The fifth passage leukemia was carried by cell-graft through 3 successive transplantations into adult C3H mice. The leukemic C3H mouse from the third transplantation was then used as donor from which a leukemic cell suspension was prepared. After centrifugation at 3,000 rpm for 15 minutes, then at 9,500 rpm for 10 minutes, part of the final supernate was passed through Selas, porosity 02, filter candle. Three newborn C3H mice were inoculated with the filtrate and all developed leukemia at 3 months of age. Nine newborn mice (3 litters) were inoculated with the 9,500 rpm supernate; 8 developed leukemia at 2½ to 6½ months of age, and 1 is still in good health at 8 months of age.

Seventh passage. One of the leukemic males from the sixth passage with filtrate-induced leukemia was used as donor; liver, spleen and mesenteric tumor were ground, and centrifuged at 3,000 rpm for 15 minutes, then at 9,500 rpm for 5 minutes. Part of the final supernate was passed through a Selas, porosity 02, filter candle. Six C3H litters were inoculated as follows: 19 newborn mice were inoculated with the filtrate, (13 subcut., 2 intraper. and 4 intracran.) and all developed leukemia at 2½ to 3½ months of age. Seven newborn mice were inoculated subcutaneously with the 9,500 rpm supernate, and all developed leukemia at 3 months of age.

Alternate seventh passage. Two leukemic females from the sixth passage were used as donors. Leukemic cell suspensions, prepared separately, were centrifuged at 3,000 rpm for 15 minutes, then at 9,500 rpm for 10 minutes. Part of the second supernate was passed through Selas, porosity 02, filter candles. Two extracts were prepared. The first extract was inoculated into 2 C3H litters, as follows: 2 newborn C3H mice were inoculated with the 9,500 rpm supernate, and both developed leukemia at 1 month of age. Five newborn C3H mice were inoculated with the filtrate, and all developed leukemia (2 at 2 months, 1 at 3, and the remaining 2 at 5 and 5½ months of age). The second extract, a filtrate, was inoculated into 3 newborn C3H mice, and all developed leukemia at 2½ months of age.

Eighth passage. Several extracts have been recently prepared from seventh-passage-donors, and inoculated into newborn C3H mice. Only one of these experiments has been completed: an extract prepared from a 2-month-

old leukemic female of the preceding passage was centrifuged at 3,000 rpm for 15 minutes, then at 9,500 rpm for 5 minutes. The final supernate was inoculated into 13 newborn C3H mice (5 litters), and all developed leukemia at 3 to 3½ months of age.

Preservation of leukemic agent at –70°C. Leukemic cell suspensions prepared in the usual manner from livers, spleen, mesenteric and mediastinal tumors from leukemic donors were placed in glass ampoules; after sealing, the glass ampoules were quickly frozen in carbon dioxide ice, then placed in metal boxes surrounded by blocks of carbon dioxide ice in an ice chest, and preserved at –70°C. The extracts proved active after preservation at –70°C for periods tested thus far up to 4½ months. Defrosted and inoculated into newborn C3H mice, such extracts induced either leukemia, parotid gland tumors, and/or subcutaneous sarcomas, with no appreciable prolongation in latency period. The following experiment will serve as an example: On Apr. 18, 1956, a 9½-month-old Ak female, No. 189, which developed spontaneous leukemia, was sacrificed, and from leukemic organs a cell suspension of 20% concentration was prepared, then placed in an ampoule, sealed, and frozen at –70°C. After 53 days, the ampoule was removed and defrosted in tap water within a few minutes. The ampoule was then opened, its contents aspirated with syringe, placed in centrifuge tube, and centrifuged at 3,000 rpm for 15 minutes and then at 9,500 rpm for 5 minutes. The second supernate was inoculated into a C3H litter, consisting of 6 mice; 5 developed leukemia at ages varying 4½ to 7 months.

That the leukemic agent could also be preserved at –70°C as a cell-free extract, was evident from the following experiment: On Aug. 21, 1956, a cell suspension prepared from a leukemic sixth-passage-donor was centrifuged at 3,000 rpm for 15 minutes and then at 9,500 rpm for 5 minutes. Three ml of the final 9,500 rpm supernate were then sealed in an ampoule, and frozen in carbon dioxide ice at –70°C. After 46 days, the ampoule was defrosted within a few minutes, and the extract, without any further treatment, was injected into 2 newborn C3H litters. Five mice were inoculated in the first litter, and all developed leukemia at 3 to 3½ months of age. Three mice were injected in the second litter, and 2, thus far, have developed leukemia at 3½ months of age.

TABLE I. Results of Inoculation of Cell-Free (Centrifuged or Filtered) Extracts from Leukemic Passage A Strain into Newborn* C3H Mice.

Passage No.	No. extr. inoc.	No. litters inoc.	No. mice inoc.	No. dev. leukemia	Avg age leukemia dev. (mo)	Leukemia incidence (%)
1†	1	1	7	6	6	86
2	1	1	3	3	3	100
3	1	1	5	2	7	40
4	1	4	7	3‡	6	43(86)‡
5	1	2	5	5	4½	100
6	3	5	12	11	7	92
7	4	10	37	37	3	100
8	1	5	13	13	3	100

* Most mice were < 12 hr old, many < 6 hr, none > 16 hr at time of inoc.

† First extract prepared from 7½-mo-old Ak female with spontaneous leukemia.

‡ Three additional mice in this group developed bilateral parotid tumors at 5 mo of age. Total incidence of leukemia and tumors, 86%.

In another experiment, a leukemic filtrate prepared on Aug. 21, 1956 from a 6th passage donor, was sealed in an ampoule, frozen at –70°C, and then kept in carbon dioxide ice for 103 days. On Dec. 4, 1956, the ampoule was defrosted within a few minutes in tap water, and its contents, without any further treatment, inoculated into a newborn C3H(f) litter. Of 6 mice, 3 have already developed leukemia at 2½ months of age; the remaining 3, now less than 3 months old, are still in good health.

Discussion. During the course of recent experiments, the observation was made that one line of the leukemic agent, that originated in a leukemic Ak mouse, and has been since carried through several successive cell-free passages, induced consistently a high incidence of leukemia, following inoculation into newborn C3H mice. A standardized method of preparing the extract as either a 9,500 rpm supernate, or Selas, porosity 02, filtrate, was adopted; such extracts were inoculated into newborn C3H mice, usually by subcutaneous route, occasionally intraperitoneally or in-

tracranially. Most of the inoculated mice developed acute, generalized, lymphatic leukemia at 2½ to 3½ months of age (Table I). Filtered extracts appeared as potent as centrifuged (9,500 rpm) supernate; subcutaneous, intraperitoneal and intracranial routes of inoculation appeared equally effective. Furthermore, the potency of the agent did not appear to be impaired by preservation at –70°C for 46 days to 4½ months.

The results thus far obtained with the mouse leukemia passage agent, could be compared to studies with the Rous sarcoma virus recently reported by Bryan and his associates (8). At first, when the filterable chicken tumor was discovered by Rous(9), it was not always possible to duplicate cell-free transmission using any tumor-carrying-chicken as a donor for the preparation of the filtrate. Gradually, it has been learned that different tumors contain different amounts of infective virus; cell-free passage of the Rous sarcoma virus from one host to another gradually increased the potency of the extracts. It is now possible to induce sarcomas in chicks with cell-free extracts in 100% of cases after a short latency of only 1 to 2 weeks(8). Ampoules containing the chicken tumor agent can be kept at –70°C and used as needed for inoculation of 3-week-old chicks of a susceptible strain. With chicken sarcoma and chicken myeloblastosis(10), the selection of a susceptible strain of hosts for inoculation of the virus is as important as in mouse leukemia(4). It is equally important, however, to use potent extracts for inoculation.

It should also be emphasized that the cell-free, centrifuged or filtered extracts, prepared from the highly potent passage strain A of mouse leukemia here reported proved to be consistently pathogenic only when inoculated into newborn C3H mice. These mice were in most instances less than 12, never more than 16 hours old at time of inoculation. In each experiment, young adult (1½ to 3 months old) C3H mice have been also routinely inoculated. Thirty-five filtered extracts were prepared, each from a different leukemic donor, and inoculated into a total of 116 young adult (approximately 2-month-old) C3H mice. Only 4 out of 94 adult C3H mice inoculated with the leukemic filtrates intraperitoneally (0.5 to 1 ml each), and 1 out of 22 inoculated intracranially (0.1 to 0.2 ml each), developed leukemia.

Summary. 1. A strain of a filterable mouse leukemia agent has been developed from a spontaneous Ak leukemia through several successive cell-free inoculations of newborn C3H mice. 2. This agent induced acute, generalized leukemia within 2½ to 3½ months in 40 to 100% of the inoculated newborn C3H mice (Bittner substrain). 3. Newborn mice had to be inoculated; adult C3H mice were with few exceptions resistant to inoculation of the cell-free agent. 4. The leukemic agent could be preserved at –70°C from 46 days to 5½ months, with no apparent loss of infectivity.

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