# Transmission of Ak Leukemic Agent into Newborn Mice of the C57 Brown/cd Inbred Line.* (21217)

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In previous experiments, cell suspensions (1,2) or centrifuged or filtered (3-7), extracts prepared from livers, spleens, and lymphatic tumors of leukemic Ak (subline Ak-n) mice, were inoculated into newborn mice of the C3H inbred line. These Ak leukemic cells multiplied rapidly, causing leukemia within 2 to 4 weeks, but did not change their genetic specificity, since they could be readily grafted back to the Ak donors, but not to adult hosts of the recipient C3H line (5). On the other hand, when either centrifuged or filtered Ak leukemic extracts were inoculated into newborn C3H mice, leukemia, which developed in some of the inoculated animals after a considerable interval of time (3½ to 27 months), was specific for recipient strain, and could be readily transplanted, by cell transfer, to adult mice of recipient C3H line, but only exceptionally to the donor Ak strain. These results suggested that when either centrifuged or filtered Ak leukemic extracts were used for inoculation of newborn C3H mice, the resulting leukemia was caused by a cell-free, transmissible agent, acting on cells of its new host.

The purpose of this report was to determine whether mice of another low-leukemic inbred line could also be used for inoculation with Ak leukemic cells, and particularly with either centrifuged or filtered Ak leukemic extracts. The use of a different recipient strain for inoculation of Ak leukemic extracts appeared indicated because among C3H mice that had been inoculated with either filtered (6,7), or centrifuged and then diluted (8), Ak leukemic extracts, some developed typical leukemia, others, however, unexpectedly developed salivary (parotid) gland carcinomas. Without discussing the possible origin of these salivary gland tumors, it appeared of interest to determine whether another strain of recipient mice might also prove susceptible, under similar experimental conditions, to development of either leukemia, or salivary gland carcinomas. For that purpose, mice of the inbred strain C57 brown, subline cd, were used for inoculations.

*Materials and methods. Leukemic extracts.* Six- to 11-months-old Ak (subline Ak-n) female mice that developed leukemia spontaneously, or, in a few instances, young adult (2- to 3-months old) Ak males and females that developed leukemia as a result of implantation of Ak leukemic cells, were used as donors of the leukemic organs. Under ether anesthesia, livers, spleens, mesenteric tumors and peripheral lymph glands were removed aseptically, and ground with sterile, physiological sodium chloride solution, at 0°C, to obtain suspensions of 20% concentration. These cell suspensions were used immediately for inoculation. In the series in which centrifuged extracts were used, the leukemic cell suspensions, prepared as described above, were centrifuged at 3000 rpm (1400 x g) for 15 minutes at 0°C; the supernatant was removed and centrifuged at 9500 rpm (7000 x g) at 0°C for 10 minutes. The final supernate was then placed in glass tubes chilled to 0°C. This supernate was passed through Selas (porosity 02 or 03) microporous porcelain filter candles, under vacuum pressure of 20 to 25 mm of mercury. Each filter was tested, before and after filtration, and found to be impervious to *E. coli.* (In 2 experiments, the centrifuged leukemic extracts (15 cc) were mixed with one cc of a 1:500 dilution of fresh broth culture of *E. coli,* and then passed through Selas filter candles. The filters retained the *E. coli,* since the filtrates were sterile.) The filtered extracts were immediately placed in sterile glass tubes at 0°C. The tubes containing centrifuged, or filtered, leukemic extracts were immersed in larger glass containers filled with ice cubes and water, and kept in refrig-

* Aided, in part, by a grant from the Damon Runyon Memorial Fund for Cancer Research.

erators. The extracts were thus kept at 0°C for periods not exceeding 24 hours, prior to inoculation. All extracts were ice-cold when injected. The inoculations were subcutaneous, approximately 0.1 cc injected into each newborn mouse. In most experiments, leukemic extracts heated in electric water bath at 67°C to 80°C for 30 to 60 minutes, were inoculated into litter-mate infant mice; in one experiment 3 litter-mates were left untreated.

*Animals.* Mice of the low-mammary-tumor inbred strain C57 brown, subline cd (C57BR/cd) were used as test animals for inoculations of the Ak leukemic extracts, because this inbred line is essentially free from spontaneous leukemia. According to information from the Jackson Memorial Laboratory(9), about 4% of very old animals of this line have been observed to develop spontaneous neoplasms of reticular tissue, usually lymphosarcomas. Six couples of 78th and 79th generations of C57BR/cd inbred line of mice were obtained in 1952 through the courtesy of Dr. E. Russell of the R. B. Jackson Memorial Laboratory, Bar Harbor, Me., and from this nucleus a small colony raised in this laboratory by brother-to-sister mating. Within a few months a sufficient number of descendant mice became available so that couples of brothers and sisters could be placed in individual cages. Mice born to these couples in successive litters were used for inoculations of leukemic extracts. Additional young adult C57BR/cd mice obtained from the Jackson Memorial Laboratory in 1952 and 1953, were not used for breeding, but only for those experiments which dealt with the transplantation of leukemic cells. We have observed neither spontaneous leukemia nor any other tumors among our untreated mice of the C57BR/cd inbred line. Fifty-three untreated mice died when 6 to 13 months old, without leukemia or tumors. Forty-seven untreated males and females (7 to 20 months old) are now in good health.

*Experimental. Results of inoculation of Ak leukemic cells.* Newborn C57BR/cd mice were susceptible to implanted Ak leukemic cells. Of 33 mice, inoculated when less than 2 days old, 29 developed subcutaneous tumors at the site of inoculation, followed by generalized leukemia, in most instances within 3 to 4 weeks. Of 34 suckling infant mice inoculated when 4 to 12 days old, only 8 developed leukemia. Of 27 suckling infants inoculated when 14 days old, 6 developed leukemia. When 20- to 24-day-old mice were inoculated, no leukemia resulted (Table I), but 2 of the 17 inoculated mice developed subcutaneous tumors within 2 weeks at site of inoculation; these tumors persisted for several days, then regressed completely. When 53 adult (2 to 11 months old) males and females were inoculated either intraperitoneally (41 mice) or subcutaneously (12 mice) with large doses (0.5 to 5 cc) of Ak leukemic cell suspensions, none developed leukemia.

TABLE I. Results of Inoculation of Ak Leukemic Cell Suspensions into C57BR/cd Infant Mice.

| Route of inoc. | Dose, cc | Age at inoc., days | No. of mice inoc. | No. of mice devel. leuk. | Incub. time, days |
|---|---|---|---|---|---|
| s.c. | .1 –.2 | <1–<2 | 33 | 29 | 15–63 |
| s.c. or i.p. | .15–.2 | 4–12 | 34 | 8 | 15–49 |
| " | .15–.5 | 14 | 27 | 6 | 15 |
| " | .15–.5 | 20–24 | 17 | 0* | |

* In 2 mice small subcut. tumors developed at site of inoculation within 2 weeks, but later regressed spontaneously.

*Further transplantation experiments.* In 4 experiments, the leukemic tumors in suckling mice following implantation of Ak leukemic cells, were used for preparation of cell suspensions, which were then implanted intraperitoneally into adult mice (2 to 4 months old) of the donor Ak strain (14 mice) and into those of the recipient C57BR/cd inbred line (14 mice). The 14 Ak mice developed leukemia within 2 to 3 weeks following inoculation, whereas the 14 C57BR/cd mice remained in good health. These results indicated that the Ak leukemic cells, which had been implanted into suckling C57BR/cd mice, retained their genetic Ak properties, even though growing and multiplying in a foreign (C57BR/cd) host. Accordingly, they were readily transplanted to adult mice of the donor Ak strain, but could not be transferred, by cell inoculation, into adult hosts of the recipient C57BR/cd inbred line(5).

*Inoculation of centrifuged or filtered Ak leukemic extracts into newborn C57BR/cd mice.* The centrifuged (7000 x g) Ak leu-

kemic extracts were inoculated into 71 newborn C57BR/cd infant mice in 16 experiments, average age 6 hours.† Of the 71 inoculated mice, 31 developed typical generalized leukemia (44%) after a latent period of 2½ to 16½ months (average age 9.6 months). In another series, filtered (through Selas filter candles) Ak leukemic extracts were used for inoculation of newborn C57BR/cd mice in 9 experiments, average age 7 hours† at time of inoculation. Of 42 inoculated mice, 14 developed typical generalized leukemia (33%) after a latent period of 10 to 14 months (average age 12.3 months). These mice developed pea-size peripheral lymph nodes, particularly prominent around the neck and in the axillary pits; they had enormous spleens and livers, large cylindrical, white and friable mesenteric, and frequently also mediastinal, lymphoid tumors. There was no evidence of any new growth at site of initial subcutaneous inoculation. The bone marrow, on smears, showed infiltration with lymphoblasts. Microscopic sections of livers showed infiltration with leukemic cells, particularly prominent around the larger vessels. The white cell blood count varied, in most instances, from 17250 to 209600/cu mm. Smears showed abnormal, immature white cells (1% to 10%) and predominance of lymphocytes (55% to 83%). Occasionally(2) the peripheral white blood count appeared normal, even though the sacrificed mice had a typical leukemia (large spleens and livers, and enlarged peripheral lymph nodes). As controls, in both experimental series, 43 newborn litter-mates were inoculated simultaneously with heated (67° to 80°C for ½ to one hour) Ak leukemic extracts; 3 litter-mates were untreated. Of 30 control litter-mates, injected with centrifuged heated extracts, 9 died at 9½ to 15 months of age without signs of leukemia; the remaining 21 are in good health, 15½ to 19 months old (average 18 months). Of the 16 control litter-mates injected with filtered heated extracts, or non-treated, 7 died without signs of leukemia when 9½ to 15 months old; the remaining 9 are well, at 11 to 17 months of age (average 14 months).

*Further transplantation of leukemia developing in C57BR/cd mice after inoculation of either centrifuged or filtered Ak leukemic extracts.* Leukemic cell suspensions prepared from livers, spleens and mesenteric tumors of those C57BR/cd mice that had developed leukemia as a result of inoculation of either centrifuged or filtered Ak leukemic extracts, were inoculated intraperitoneally (0.25 to 0.5 cc) into adult, *i.e.,* more than 2 months old, mice of the C57BR/cd, Ak, C58, and C3H inbred lines. In each of 15 individual experiments, a different leukemic C57BR/cd donor mouse was used for the preparation of leukemic cell suspensions. Thirty-seven C57BR/cd mice were inoculated, and 30 developed leukemia after average incubation time of 31 days. Only 4 out of 35 Ak females developed leukemia 77 days after inoculation. None of the inoculated 12 C58, or 19 C3H mice developed leukemia. Thus, the C57BR/cd leukemic cell suspensions could, in most instances, be readily transplanted to adult mice of the C57BR/cd inbred line, but not to other strains; they could not be transplanted back to adult mice of the donor Ak strain. Only in a single transplantation experiment did 4 Ak females develop leukemia following implantation of the C57BR/cd leukemic cells. In these 4 mice, however, leukemia developed after a latency of 77 days; these females were then almost 6 months old, when leukemia may occur spontaneously. It is therefore questionable whether these 4 Ak mice developed leukemia spontaneously, or as a result of implantation of the C57BR/cd leukemic cells.

*Discussion.* Suckling, less than 2-day-old mice of the C57BR/cd inbred line were highly susceptible to inoculation of Ak leukemic cells; mice 4 to 14 days old were, in some instances, still susceptible, but the 20-day-old mice were already resistant. Adult (2 to 11 months old) C57BR/cd mice were completely resistant to inoculation of large (0.5 to 5 cc) doses of Ak leukemic cells. Following inocu-

† Only newborn C57BR/cd mice a few hours old were used for inoculation of centrifuged or filtered leukemic extracts, because previous experiments (3-5) suggested that susceptibility of newborn mice to inoculation of cell-free Ak leukemic extracts diminishes rapidly after the first 12 hours of life.

lation of Ak leukemic cells into newborn C57BR/cd mice, a leukemic tumor (lymphosarcoma) usually appeared at site of inoculation, followed within 2 to 4 weeks, by a generalized leukemia. Such transplanted leukemia consisted of Ak cells. The new foreign host served as a live medium, sufficiently susceptible to provide favorable conditions for multiplication of implanted Ak leukemic cells. This was evident from further experiments which showed that such transplanted leukemic tumors could be readily grafted back into adult mice of the Ak donor strain, but not usually into those of the C57BR/cd line.

The results were different when cell-free Ak leukemic extracts were used for inoculation. Thus, when either centrifuged or filtered Ak leukemic extracts were inoculated into newborn C57BR/cd mice (average age 6 to 7 hours†) generalized leukemia developed after a considerable delay, varying from 2½ to 16½ months, with no evidence of new growth at site of initial subcutaneous inoculation. Leukemic cell suspensions prepared from livers and spleens of such animals, produced acute leukemia when transplanted into adult mice of the same (C57BR/cd) strain, but could not be grafted, with rare exceptions, back to adult mice of the Ak inbred line.

These findings suggest that following inoculation of centrifuged or filtered Ak leukemic extracts into newborn C57BR/cd mice, the cell-free agent, introduced into the recipient strain, acted on susceptible cells of its new host in such a manner that after several months, leukemia resulted. This induced leukemia consisted, however, of its new host's own cells, *i.e.*, it was a C57BR/cd leukemia.

It has not yet been determined whether mice of the C57BR/cd inbred line, once inoculated with the Ak leukemic extracts, will be sufficiently susceptible to pass the acquired leukemic agent to their own untreated offspring, and then from one generation to another. Twenty-two males and females of this line, that had been inoculated with Ak leukemic extracts within a few hours after birth, were mated after they reached sexual maturity, and when in good health. Among their 147 untreated offspring, only 10 have thus far developed leukemia at 7 to 17 months of age. The remaining 137 mice are now only 12 to 17 months old. Additional time is needed to complete this series of observations. These preliminary results, though incomplete, indicate that mice of the C57BR/cd inbred line, because of limited susceptibility, may occasionally transmit to their offspring an experimentally acquired Ak leukemic agent.

When filtered (Berkefeld, N, or Selas 03) (6,7), or centrifuged and then diluted(8), Ak leukemic extracts were inoculated into newborn C3H mice, some animals developed leukemia, and others, parotid gland carcinomas. When, however, filtered or centrifuged, Ak leukemic extracts were inoculated into C57BR/cd mice, typical generalized leukemia appeared in some inoculated animals, but none developed parotid gland carcinomas. Although the true cause of development of salivary gland carcinomas among C3H mice, inoculated with Ak leukemic extracts, has not yet been fully clarified, it now appears that these tumors can be induced only in certain strains of mice, such as the C3H inbred line. Thus, leukemic extracts which readily induced salivary gland carcinomas in C3H mice, did not cause development of such tumors when injected under apparently identical experimental conditions into mice of the C57BR/cd inbred line.

The results reported in this study are of interest because they confirm the presence of a filterable and thermolabile, cell-free agent in the Ak leukemic extracts. Whereas it was shown that this agent is pathogenic for C3H mice(1-8), and also for mice of the C57 black inbred line,‡ causing, when inoculated under

‡ In a preliminary series, 44 C57 black mice were inoculated when less than 12 hours old with Ak leukemic extracts centrifuged at either 3000 rpm (1400 x g) or at 9500 rpm (7000 x g), and 17 of them (39%) developed typical generalized leukemia at ages varying from 11½ to 22 months (average 17 months). Since less than 10% of untreated very old mice of the C57 black inbred line developed spontaneously lymphatic tumors(10), these data suggest that the C57 black mice developed leukemia as a result of inoculation with the Ak leukemic agent. Because of the relatively high incidence of spontaneous lymphatic tumors in mice of the C57 black line, however, we discontinued using these mice for inoculations with the Ak leukemic ex-

certain conditions, the development of leukemia, this study shows that the same agent is also pathogenic for newborn mice of the strain C57BR/cd. The Ak leukemic agent is therefore apparently pathogenic for mice of certain susceptible foreign strains, such as the C3H, C57BR/cd, and C57 black, provided that it is inoculated into the new hosts within a few hours after birth.

Since similar observations have been made with filtered extracts prepared from C58 leukemic donors(8,11), it now appears that both the Ak and the C58 spontaneous mouse leukemias are caused by filterable, thermolabile agents which, under certain conditions, can also be transmitted, by inoculation, to other strains of mice. In nature, however, these agents apparently would be transmitted in certain families of mice from one generation to another directly through the embryos(3,4,11). From an etiological and epidemiological point of view, mouse leukemia would be essentially similar to chicken lymphomatosis, also caused by a filterable agent(12) transmitted from one generation to another directly through the embryos(13). Since these causative agents are presumably of viral nature, both mouse leukemia(14), and chicken lymphomatosis (15) could be considered as "egg-borne" virus diseases.

*Summary.* 1. Cell suspensions prepared from Ak leukemic donors were inoculated into newborn C57BR/cd mice. Of 33 mice inoculated when less than 2 days old, 29 developed leukemia within 2 to 4 weeks. Of 61 inoculated when less than 14 days old, 14 developed leukemia, but of 17 injected at 20 to 24 days of age, none developed leukemia. Fifty-three adult C57BR/cd mice over 2 months old were inoculated with large doses of Ak leukemic cell suspensions, and all were resistant. 2. Leukemia developing in suckling C57BR/cd mice as a result of implantation of Ak leukemic cells, could be readily grafted to adult mice of the donor Ak strain, but not into recipient C57BR/cd line. 3. Centrifuged (7000 x g) Ak leukemic extracts were inoculated into 71 newborn C57BR/cd mice (average age 6 hours), and 44% of them developed leukemia at average age of 9.6 months. Filtered (Selas) Ak leukemic extracts were inoculated into 42 C57BR/cd mice at average age of 7 hours, and 33% of them developed leukemia at average age of 12.3 months. 4. Forty-three litter-mates serving as controls, were inoculated simultaneously with heated (67° to 80°C for ½ to one hour) Ak leukemic extracts, and 3 were untreated, but none developed leukemia. 5. Cell suspensions prepared from those C57BR/cd mice that developed leukemia as a result of inoculation of either centrifuged or filtered Ak leukemic extracts, reproduced, in most instances, within a few weeks, acute leukemia, when transplanted into adult mice of the C57BR/cd (recipient) line, but could not be grafted, with rare exceptions, to adult mice of the Ak (donor) strain. 6. When, in previous experiments, filtered Ak leukemic extracts were inoculated into newborn C3H mice, some of the inoculated animals developed leukemia, and others developed parotid gland carcinomas. When, however, filtered Ak leukemic extracts were inoculated into C57BR/cd mice, typical leukemia developed in some of them, but none developed salivary gland carcinoma. 7. Twenty-two C57BR/cd males and females, that had been inoculated with Ak leukemic extracts within a few hours after birth, were mated after they reached sexual maturity, and in good health. Among their 147 untreated offspring, 10 have thus far developed leukemia at 7 to 17 months, suggesting transmission of leukemic agent from inoculated parents to untreated offspring.

tracts. Instead, mice of the C57 brown strain, subline cd, (C57BR/cd), essentially free from spontaneous leukemia, have been selected for this purpose.

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Received June 22, 1954. P.S.E.B.M., 1954, v86.

## Phosphorylation in Cardiac Muscle from Failing and Unfailing Heart-Lung Preparations.*† (21218)

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(Introduced by M. H. Seevers.)

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Little is known about the sequence of biochemical events that leads to failure of heart muscle either in the intact animal or in isolated preparations. In an attempt to elucidate the cause of failure, most of the studies have been centered around the various enzymatic steps in the breakdown of carbohydrate and the concomitant generation of energy, primarily because more is known of these biochemical pathways. One approach to the problem of whether or not failure is associated with a defect in energy production has been the analysis of the high-energy phosphate stores of the normal and failing heart(1,2). The results of these studies have indicated that in failure, cardiac muscle retains its normal level of phosphocreatine and adenosine triphosphate. However, the level of the high-energy phosphate compounds of the heart is in reality merely the balance between the generation and the utilization of these compounds. Thus, an inhibition of both generation and utilization might still result in normal levels of high-energy compounds. It was therefore decided to study in a more direct way the ability of failed and unfailed cardiac muscle to generate energy-rich bonds.

*Methods.* In the control group of animals, mongrel dogs were anesthetized and immediately sacrificed, or the failed heart from the dog heart-lung preparation was employed. The animals sacrificed immediately were anesthetized with ether or pentobarbital, while preliminary operations on the heart-lung preparation were performed under pentobarbital anesthesia. The failed hearts in all experiments were obtained from the dog heart-lung, prepared by a modification of the Krayer-Mendez method(3). Failure in these animals was either spontaneous or pentobarbital induced. The competance index as described by Wollenberger(1) was used to evaluate the degree of failure. Those preparations having indices of from 0.8-1.0 were considered to be unfailed, while those with indices of from 0.0-0.2 were taken to be failed. In both failing and unfailing animals the left ventricle was rapidly excised and placed in 0.25 M sucrose solution. Mitochondria were then prepared by a modification of the method of Langemann *et al.*(4). Operations from this point on were carried out in ice baths in order to maintain a temperature of from 3-5°C. The tissue was then minced, rapidly weighed and a 10% homogenate prepared in isotonic sucrose using an all-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged for 5 minutes at 700 x g in a refrigerated centrifuge. The residue was discarded and the supernatant fluid was centrifuged at 12000 x g for 15 minutes. The resulting residue was resuspended in 0.25 M sucrose and recentrifuged at the same speed. The final

* Supported in part by a grant from the Michigan Heart Assn.

† Part of this paper was presented at the Am. Soc. for Pharmacol. and Exp. Therap., New Haven, Sept. 1953.