A massive dose of approximately 1.5×10^8 viable cells of Leptotrichia was injected intravenously into each of 18 rabbits. All survived for 28 days. *L. dentium* was isolated from the blood of these animals subcultured one hour post-inoculation. Consistently negative cultures were obtained from samples taken 2 hours post-inoculation. Six rabbits which received a similar dose by the subcutaneous route also survived, but developed an ulcerative lesion approximately 14 mm in diameter. The lesions were slightly raised, erythematous, peripherally fluctuant and centrally indurated. Materials expressed from these were whitish and odorless. Subcultures on various media from this material were negative. The lesions appeared within 24 hours post-inoculation and healed in approximately 2 weeks.

Summary. 1. *Leptotrichia dentium* organisms have been isolated from *materia alba* by plating out on Brain Liver Heart agar containing 7 μg/ml polymyxin. The efficacy of this medium resides in its content of polymyxin which permits growth of *L. dentium* and inhibits growth of some of the other forms commonly present in such specimens. 2. A defined medium is described which permits optimal growth of *L. dentium*. The medium is composed of Casamino acids, tryptophane, cystine, glucose and 5 vitamins. Of the vitamins, riboflavin and nicotinic acid are essential, whereas calcium pantothenate, biotin, and nicotinic acid are stimulatory. 3. *L. dentium* appeared to be non-pathogenic for rabbits. Animals given intravenously a massive dose of viable cells (1.5×10^8) survived for 28 days. Animals given equivalent dose by the subcutaneous route also survived but developed a small ulcerative lesion at site of inoculation which healed in approximately 2 weeks.

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Transmission of Mouse Leukemia Virus Through Milk of Virus-Injected C3H Female Mice.* (27350)

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We reported(1) experiments in which C3H mice that had been inoculated with a highly potent leukemic virus (passage A) transmitted this virus to their offspring. All non-treated offspring born to, and raised by, virus-injected mothers developed leukemia, and the disease appeared after a relatively short latency of 4½ to 8 months. It was immaterial whether the father was inoculated, provided that the mother received inoculation of

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the leukemic virus. On the other hand, no leukemia was recorded at the time our paper was prepared(1) among the 31 offspring born to non-injected mothers and virus-inoculated fathers; later however, 7 mice (23%) in this group developed leukemia at 14 to 17 months of age, suggesting that the leukemic virus could also be transmitted, though to a lesser extent, from one generation to another, through the father. This observation was also consistent with results of previous similar experiments (Gross, unpublished).

This striking difference in incidence and latency of leukemia developing in the offspring, depending on the parent inoculated, *i.e.*, the high incidence and early development of leukemia in offspring born to the virus-injected mothers and either virus-injected or non-injected fathers, as compared with the low incidence and delay in appearance of leukemia in offspring born to non-injected mothers and virus-injected fathers, prompted experimental studies here reported, and designed to determine whether the leukemic virus could not be transmitted through the milk of nursing mothers.

Previous experiments suggested that the leukemic virus in mice of high-leukemic inbred lines, such as Ak or C58, is transmitted, under natural conditions of life, from one generation to another directly through the embryos(2,3,4). It appeared reasonable to assume, therefore, that in our recent experiments, in which a considerably more potent leukemic virus was employed, natural transmission of the leukemic virus also occurred in a similar manner.

Experiments reported here suggest, however, that transmission through the embryos is not the only manner of natural passage of the leukemic virus from one generation of mice to another. Under certain experimental conditions the leukemic virus could be also transmitted in the milk of nursing female mice. This was accomplished when females which had been inoculated with a very potent leukemic virus were employed as foster mothers shortly before, or at the time, they developed signs of disease.

Materials and methods. The 28th to 30th cell-free serial passage A of the leukemic virus strain initially isolated from spontaneous Ak mouse leukemia, and then passed serially through newborn C3H mice(5) was employed in this study. At this passage level, the potency of this virus has been sufficiently high to induce an incidence of leukemia of approximately 98% after a latency of about 2½ months, following inoculation into suckling mice of either C3H/Bi or C57 Brown/cd inbred lines; the virus is also highly pathogenic for suckling rats(6). Filtrates (Selas 02) of 20% concentration prepared in the usual manner(5) from C3H donor mice with passage A virus-induced leukemia, were inoculated intraperitoneally (0.2 to 0.3 ml). All animals were of the Bittner subline of strain C3H, free from the mammary carcinoma virus. In our laboratory the incidence of spontaneous leukemia in mice of this strain has not exceeded during the past several years 0.5 to 1% in animals beyond 12 months of age.

Newborn C3H mice were inoculated, within a few days after birth, with passage A leukemic filtrate. After these mice reached sexual maturity, they were mated; in some experiments a virus-injected female was mated to a virus-injected male from the same litter. In other experiments, a virus-injected female was mated to a non-treated, normal C3H male of the same age, selected for that purpose from our colony of C3H mice. As soon as a litter was born to one of the virus-injected mothers, another litter was located, born at about the same time to normal, non-injected C3H mice in the colony of this strain maintained in our laboratory. In most experiments the litters were then exchanged; the litter born to the virus-injected mother was removed as soon as possible after birth, and placed with the normal, non-injected (colony) foster mother; reciprocally, the normal litter of suckling mice born to the non-injected mother was placed with the virus-injected foster mother. In some experiments, only one of the litters was transferred for foster-nursing, and the other litter was destroyed.

Experimental results. In the first experiment, a 3-day-old C3H litter was inoculated with the leukemic filtrate. Four weeks later

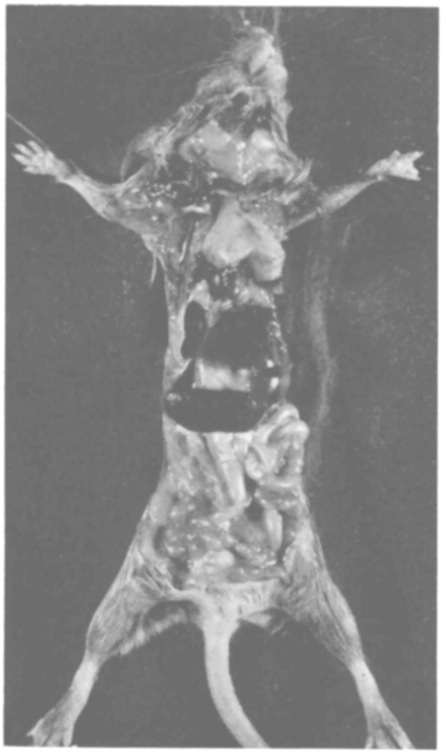

FIG. 1. This C3H female No. 260, Exp. LV 503 (Table I) was born to healthy, non-injected parents from our normal colony of C3H mice, but was foster-nursed by a virus-injected C3H female; the latter developed leukemia at time of nursing. The foster-nursed female No. 260 developed lymphatic leukemia at the age of 4½ mo. Note very large mediastinal lymphosarcoma, also a large ruptured spleen, and a moderately large oblong mesenteric tumor.

a virus-injected female (No. 581) from this litter was mated to a non-injected, healthy 4-week-old C3H male. A litter, consisting of 6 mice, born to female No. 581 one month later, was removed within 18 hours after birth from its virus-injected mother and transferred to a healthy, non-injected female No. 590, from our colony of C3H mice. Female No. 590's own litter, born at about the same time, was transferred for the purpose of foster-nursing to the virus-injected female No. 581. The latter nursed the substitute litter for 14 days, *i.e.*, until she developed advanced generalized lymphatic leukemia. It was then necessary to substitute an alternate healthy foster mother from our colony of C3H mice for additional 9 days to assure survival of the foster-nursed litter. All 6 C3H mice (4 females and 2 males) born to healthy non-injected female No. 590 from our colony, but foster-nursed for 2 weeks by the virus-injected female No. 581, developed leukemia (Fig. 1) at 4½ to 5½ months of age (Exp. LV 503, Table I). On the other hand, of the 6 mice (3 males and 3 females) born to the virus-injected female No. 581, but transferred within 18 hours after birth to a healthy foster mother, only 1 male, thus far, developed leukemia at 6 months of age (Exp. LV 494, Table II). These mice are now 6½ months old.

In the second experiment, a 4-day-old C3H litter was inoculated with a leukemic filtrate. Four weeks later a virus-injected female (No. 520) from this litter was mated to her virus-injected brother. A litter born about one month later to the virus-injected female No. 520 was transferred immediately for the purpose of foster-nursing to a healthy, normal female No. 336 from our colony of C3H mice. Female No. 336's own litter, born a few hours earlier, and consisting of 4 mice, was transferred within a few hours after birth, for the purpose of foster-nursing, to the virus-injected female No. 520. The latter nursed the substitute litter for 14 days, *i.e.*, until she developed advanced generalized lymphatic leukemia. It was then necessary to substitute an alternate foster mother from our C3H colony for additional 8 days, to assure survival of the foster-nursed litter. Of the litter born to

TABLE I. Development of Leukemia in C3H Mice Born to Normal, Non-Injected C3H Mice, but Foster-Nursed by Virus-Injected C3H Females.*

Exp. No.	Sex	No. of foster-nursed mice	No. devel. leuk.	Leuk. inc., %	Avg age leuk. devel., mo
LV 503	♀	4	4		4½
	♂	2	2		5½
LV 504	♀	1	1		3½
	♂	2	2		4½
LV 528	♀	3	3		4½
	♂	3	3		5½
LV 527	♀	2	2		4½
	♂	3	—		
LV 536	♀	3	1		4
	♂	2	2		4½
Total		25	20	80%	5

* Inoculated within a few days of birth with passage A leukemic filtrate.

TABLE II. Development of Leukemia in C3H Mice Born to Virus-Injected C3H Mothers,* but Foster-Nursed by Normal, Non-Injected C3H Females.

Exp. No.	Sex	No. of foster-nursed mice	No. devel. leuk.	Leuk. inc., %	Avg age leuk. devel., mo
LV 494	♀	3	0		
	♂	3	1		6
LV 492	♀	1	—		
	♂	3	—		
LV 512	♀	4	1		4½
	♂	1	—		
LV 507	♀	2	—		
	♂	2	—		
LV 520	♀	2	—		
	♂	2	—		
LV 510	♀	3	2		4½
	♂	1	—		
Total		27	4	15%	5

* These females were inoculated within a few days after birth with passage A leukemic filtrate, and were subsequently mated to either virus-inj., or non-inj., C3H males.

healthy female No. 336, and foster-nursed for 14 days by the virus-injected female No. 520, 3 mice (1 female and 2 males) survived and all 3 developed advanced generalized leukemia at 3½ to 4½ months of age (Exp. LV 504, Table I). Of the litter consisting of 4 mice (1 female and 3 males) born to the virus-injected female No. 520, and foster-nursed by the healthy female No. 336, none, thus far, developed leukemia; these mice are now 6 months old (Exp. LV 492, Table II).

In the third experiment, a 5-day-old C3H litter was inoculated with a leukemic filtrate. Four weeks later a virus-injected female (No. 208) was mated to her virus-injected brother. A litter born one month later to the virus-injected female No. 208 was transferred, when less than 1 day old, for foster-nursing, to healthy, normal C3H female No. 85 from our C3H colony of mice. Female No. 85's own litter, born at about the same time, and consisting of 7 mice, was transferred simultaneously for foster-nursing to virus-injected female No. 208. The latter nursed the substitute litter for 15 days, *i.e.*, until she developed advanced generalized lymphatic leukemia. It was then necessary to substitute an alternate foster mother from our colony of C3H mice for additional 8 days, to assure survival of the foster-nursed litter. Of the litter born to healthy female No. 85 and foster-nursed for 15 days by the virus-injected female No. 208, 6 mice (3 females and 3 males) survived, and all developed leukemia at 4½ to 5½ months of age (Exp. LV 528, Table I). On the other hand, of the litter consisting of 7 mice born to virus-injected female No. 85, 5 mice (4 females and 1 male) survived, and only 1 female, thus far, developed leukemia; the remaining 4 mice are now 6 months old and are still in good health (Exp. LV 512, Table II).

In the fourth experiment, a 6-day-old C3H litter was inoculated with the leukemic filtrate. Four weeks later, a virus-injected female from this litter (No. 176) was mated to her virus-injected brother. A litter consisting of 4 mice (2 females and 2 males) born to female No. 176 one month later was transferred immediately to a healthy, non-injected female No. 488 from our colony of C3H mice. At the same time, female No. 488's own litter, born a few hour earlier, was transferred for foster-nursing to the virus-injected female No. 176. The latter nursed the substitute litter until weaning, although she developed signs of leukemia during the third week of nursing and died a few days after weaning. Of the litter consisting of 8 mice born to healthy female No. 488 and foster-nursed by the virus-injected female No. 176, 5 survived, *i.e.*, 2 females and 3 males; both females developed leukemia at 4½ to 5½ months of age; the remaining 3 males are still in good health (Exp. LV 527, Table I). Of the litter consisting of 4 mice (2 females and 2 males) born to the virus-injected female No. 176, and foster-nursed by the healthy female No. 488, none, thus far, has developed leukemia; these mice are now 6 months old (Exp. LV 507, Table II).

In the fifth experiment, a 4-day-old C3H litter was inoculated with the leukemic filtrate. Four weeks later a virus-injected female from this litter (No. 283) was mated to a healthy, non-injected C3H male of about the same age. About one month later, a litter was born to the virus-injected female No. 283. Another litter born about the same time to healthy non-injected C3H female No.

224 was located in our colony of normal C3H mice. The litters were exchanged when both were less than 6 hours old. The normal litter born to healthy, non-injected female No. 224, was foster-nursed by the virus-injected female No. 283, and was then weaned; female No. 283 died from leukemia 2 days later. Of this litter, consisting of 3 females and 2 males, 1 female and both males thus far developed leukemia at 4 to 4½ months of age; the remaining 2 females are still in good health (Exp. LV 536, Table 1). Of the litter consisting of 2 females and 2 males born to the virus-injected female No. 283, and foster-nursed by healthy female No. 224, none, thus far, developed leukemia; these mice are now 6 months old (Exp. LV 520, Table II).

In the sixth experiment, a 2-day-old C3H litter was inoculated with the leukemic filtrate. Four weeks later a virus-injected female from this litter (No. 201) was mated to her virus-injected brother. A litter consisting of 6 mice, born to this female one month later, was removed when less than 15 hours old, and placed for the purpose of foster-nursing with a healthy, non-injected female No. 22 from our normal colony of C3H mice. Female No. 22's own litter, born at about the same time, was destroyed. Of the 6 mice born to the virus-injected female No. 201, and foster-nursed by the healthy female No. 22 from our C3H colony, 4 mice (3 females and 1 male) survived; 2 females of this litter developed leukemia at about 4½ months of age; the remaining 2 mice, now 5½ months old, are still in good health (Exp. LV 510, Table I).

Discussion. Earlier experiments appeared to suggest that under natural conditions of life, mouse leukemia, unlike mouse mammary carcinoma(7), is not transmitted through the milk of nursing female mice. This assumption was based on experiments performed on inbred strains of mice, such as C58 or Ak, naturally susceptible to development of spontaneous leukemia, *i.e.,* carrying a naturally transmitted virus of relatively low potency. In these early experiments, development of spontaneous leukemia could not be prevented entirely by foster-nursing of mice born to parents of high-leukemic strains (C58 or Ak) by female mice of low-leukemic lines(8,9,10, 11). In some of these experiments, however, the incidence of spontaneous leukemia was significantly reduced in offspring of the high-leukemic strain Ak, following foster-nursing of such mice by females of a low-leukemic inbred line(9,10), but incidence of leukemia increased again in the next generation of the foster-nursed mice(10). Even when Ak embryos were removed at term by Caesarean section from the womb of their Ak mother, and transferred for foster-nursing to a female of a low-leukemic strain, spontaneous leukemia developed later at the usual age in such foster-nursed Ak mice(12,13). Similar results were obtained when Ak ova were transferred to C3H females; Ak mice born from such ova and nursed by their C3H foster mothers, developed leukemia(13). The reciprocal nursing of mice of low-leukemic strains (StoLi, Af, Rf, or C3H), by foster mothers of the high-leukemic strains (C58 or Ak), failed to induce leukemia in the foster-nursed mice of the low-leukemic lines(8,9,10, 11). It appeared reasonable to conclude that the leukemic agent was not transmitted through the milk in mice of the high-leukemic inbred lines. The presence of the leukemic virus in normal Ak or C58 embryos was demonstrated in direct bio-assay experiments(2, 3,4) in which extracts prepared from such embryos induced leukemia following inoculation into newborn C3H mice. The leukemic virus in mice was therefore found to be transmissible from one generation to another through the embryos, like the virus of chicken lymphomatosis(14).

Experiments here reported suggest, however, that under certain conditions the leukemic virus may be also transmitted in the milk of nursing female mice (Table I). This observation might appear unexpected, and at variance with the results of previous studies in which, employing mice of high-leukemic and low leukemic inbred lines, the effect of reciprocal foster-nursing on incidence of spontaneous leukemia was investigated. It is quite apparent, however, that there are important differences between the earlier studies and the experiments here reported. In the earlier studies, the leukemic virus naturally

transmitted in mice of certain high-leukemic lines was of relatively low potency; furthermore, female mice employed as foster mothers were in perfect health at the time they nursed; leukemia developed in such animals usually after a considerable lapse of time, perhaps several months after they had been employed as foster mothers. In the present study, on the other hand, a leukemic virus has been employed which, although initially isolated from spontaneous Ak leukemia, increased considerably in potency following serial cell-free passage through newborn mice; it now consistently induces leukemia in practically 100% of the inoculated C3H mice after a short latency of about 2½ months. Accordingly, in our experiments, the virus-injected female mice serving as foster mothers usually developed signs of leukemia at the time they nursed, or immediately afterwards. Since in leukemic mice the virus was found to be present in relatively high concentration in blood plasma(15), it is not surprising to find evidence of presence of the virus also in the milk of nursing mice having signs of leukemic disease.

It remains to be determined whether virus-injected, but healthy, female mice can also effectively transmit the leukemic virus in their milk several months prior to development of disease. The question of quantity of the virus shed in the milk should also be considered. A sufficient dose of the virus is obviously necessary to cause disease. The virus-carrying, but healthy, female may shed some virus in milk, but possibly not in sufficient quantity to cause disease in the offspring. The leukemic mother, on the other hand, or a female mouse nursing at a time immediately preceding the development of leukemia, may shed a sufficiently large quantity of virus in her milk to cause a subsequent development of disease in the suckling offspring. These are speculative considerations, which are being investigated.

It is now apparent that the high incidence, and early development, of leukemia recently observed in non-treated C3H offspring born to, and nursed by, virus-injected C3H females (1), was probably caused by a milk-transmitted virus. No evidence has been thus far obtained, however, to indicate that development of leukemia in the offspring of virus-injected mice could be entirely prevented by foster-nursing of such animals by healthy non-injected females (Table II). Previous experiments carried out on Ak and C58 mice suggested that the leukemic virus is transmitted from one generation to another directly through the embryos(2,3,4). Accordingly, offspring of virus-injected mice are presumably infected prior to birth, although under such conditions they may carry only a relatively small quantity of virus.

In our present study, only relatively few of the mice, born to virus-injected mothers, but foster-nursed by healthy C3H females, have thus far developed leukemia (Table II). It is realized that at least some of these mice received a small amount of milk from their own mothers, before they were transferred to healthy foster mothers; this may account for some of the leukemias observed in this group of mice. On the basis of previous experiments, it is assumed, however, that even those mice that did not receive any virus in the milk also carried the leukemic virus prior to birth, since they were born to virus-injected parents. These mice are still relatively young, and they will be observed. It is anticipated that additional animals in this group will later develop leukemia. A relatively small quantity of the virus may be transmitted directly through the embryos; accordingly, disease resulting from such prenatal infection may appear after a relatively long latency, later in life.

Summary. 1. C3H female mice inoculated within a few days after birth with passage A leukemic filtrates, were subsequently mated to virus-injected, or to non-injected, C3H males. Litters born to such parents were exchanged with those born at about the same time to normal, non-injected C3H mice. 2. Out of 25 C3H mice born to normal C3H parents, but foster-nursed by virus-injected C3H females, 20 (80%) developed leukemia at 3½ to 5½ months of age. 3. Out of 27 mice born to virus-injected mothers, but foster-nursed by normal C3H females, only 4 (15%) thus far developed leukemia at 4½ to 6 months of age. 4. These results indicate

that the leukemic virus was transmitted through the milk of nursing females; the latter, however, had leukemia at least part of the time they were nursing. It remains to be determined whether healthy, but virus-carrying, females could also transmit the virus in their milk. 5. Under our experimental conditions, foster-nursing by healthy C3H females of offspring born to virus-injected C3H mothers, reduced the incidence, but did not entirely prevent, the development of leukemia.

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Erythropoietin Assays Using Iron^{59} Incorporation into Blood and Spleen of the Polycythemic Mouse. (27351)

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The presence of a specific humoral factor stimulating erythropoiesis (erythropoietin, erythropoiesis stimulating factor, ESF) has been confirmed and measured by a number of bioassay technics(1). These have usually involved suppression of erythropoiesis in the assay animal by hypophysectomy, starvation, dehydration or induced polycythemia(1,2). Transfusion-induced polycythemia has the advantages that it is more physiologic than other means and that suppression can be maintained indefinitely. Jacobson *et al.*(3) have used reticulocyte response in the transfused mouse as a semi-quantitative assay technic. In an effort to find a sensitive assay system for ESF requiring small amounts of test material, we have investigated the erythropoietic response of the transfused polycythemic mouse to injections of ESF-rich serum as measured by the incorporation of iron^{59} into the blood and spleen, a normal hematopoietic organ in the mouse.

Methods. Female NIH strain mice weighing 18-22 g were injected intraperitoneally on Day 1 with 0.7 ml of an 80% suspension of homologous red blood cells which had been collected in acid citrate-dextrose solution and concentrated by centrifugation. On Day 3 they were injected with a further 0.4-0.8 ml of 80% RBC suspension and distributed into groups of 8 animals. Each animal was injected subcutaneously with 0.5 ml doses of test material on Day 3 and Day 4. Saline dilutions of the serum of a patient with aplastic anemia were used as stimulus for dose- and time-response data. This serum was found by comparison with a distributed standard* to contain 12 cobalt units of ESF as defined by Schlueter *et al.*(4) per ml. Animals injected with normal saline were the negative controls.

On day 5, 0.5 μc of Fe^{59} citrate in 0.1 ml of normal saline was injected intraperitone-

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