ance(7). There was no evidence of cortical necrosis or hemorrhage in the deficient mouse as has been reported for the rat. Furthermore, the changes occurring were more gradual in their appearance than in the case of the rat(8). Starvation caused complete exhaustion of sudanophilic and steroid materials from the zona fasciculata as shown in Fig. 4. In contrast, the zona glomerulosa of these animals retained some sudanophilic lipids but was practically depleted of ketosteroids. This would suggest that the physiological demands upon the adrenal cortex were much greater in starvation than in pantothenic acid deficiency.

The spleen weight of mice increased during the deficiency when compared with the controls but the amount of white pulp decreased significantly by the end of the sixth week as shown by areal analysis of sections. Histological examinations of spleen tissue from deficient mice indicated the following: (1) germ centers in the white pulp were rarely visible, (2) there were relatively few lymphocytes, and (3) lymphocytolysis was apparent as shown by the pycnotic nuclei. Furthermore, there was no indication of cell division. These changes may be correlated with the occurrence of the significant lymphopenia as shown in Table I. By the end of the seventh week the white pulp of the spleen was similar in appearance to that of the 6-week control in that the germ centers were active and the amount of white pulp had increased.

During pantothenic acid deficiency in the adult mouse, the thymus gland underwent involution and this was accompanied by an apparent increase in sudanophilic lipid in the interlobular and subcapsular connective tissues. By the seventh week there was a marked decrease in the number of lymphocytes in the thymus and as a result, the reticular network of the gland was much more conspicuous than in control animals. Dougherty and White have shown a direct relationship between the degree of involution of lymphoid tissue and the amount of corticosterone secreted(2).

Summary. Adult male mice placed on a pantothenate-deficient diet developed a lymphopenia followed by lymphocytosis when compared to control animals. The adrenal cortex showed hypertrophy and simultaneous, gradual depletion of lipid material, including ketosteroids, from the zona fasciculata. By the seventh week the zona fasciculata was nearly depleted of its hormone. In deficient mice, the thymus atrophied and there was a gradual enlargement of the spleen. Starvation caused a rapid depletion of the lipid, including the ketosteroids, from the zona fasciculata. The zona glomerulosa remained unchanged in pantothenic acid deficiency whereas in starvation it was depleted of steroid material but retained some sudanophilic lipids.

Received November 14, 1950. P.S.E.B.M., 1951, v76.

7. Deane, H. W., and Greep, R. O., *Am. J. Anat.*, 1946, v79, 117.

8. Deane, H. W., and McKibbin, J. M., *Endocrinology*, 1946, v38, 385.

"Spontaneous" Leukemia Developing in C3H Mice Following Inoculation, In Infancy, with AK-Leukemic Extracts, or AK-Embryos.* (18379)

LUDWIK GROSS.

From the Cancer Research Unit, Veterans Administration Hospital, Bronx, N. Y.

The purpose of this study was to determine whether "spontaneous" leukemia could be prompted to develop in middle aged mice, of a line known to be essentially free from this disease, by inoculating such animals, in their early infancy, with extracts prepared from mouse leukemia, or with cell suspensions prepared from embryos removed from the uterus

* Reviewed in the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the author are the result of his own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

of a mouse of a leukemic inbred line.

Materials. Animals. It was necessary for this study to use animals having a family record essentially free from leukemia. Mice of the C3H line appeared to be suitable for this purpose because they rarely, if ever, developed spontaneous leukemia(1). In our colony of C3H mice we have observed thus far a total of 2,540 females and 2,000 males and we have seen only 3 cases of spontaneous leukemia among these mice. Because, however, females of the C3H line develop mammary carcinomas, mice of a foster nursed C3H subline have been used for this study. A colony of such mice has been raised in this laboratory from a litter of C3H mice that had been nursed by a C57 female. These mice have been designated in this study by the symbol C3H (f). Up to the time of this writing we have observed 745 females and 432 males of this line through 16 successive generations, and there has been thus far no case of spontaneous leukemia among these animals (1).

Leukemic extracts. Mice of the Ak line that developed leukemia either spontaneously, or as a result of inoculation with a transplanted strain of leukemia, were sacrificed by ether inhalation, and fragments of liver, spleen, and enlarged lymph nodes, as well as small pieces of a mesenteric tumor regularly found in such animals, were removed aseptically. The leukemic tissues were weighed, then placed in a sterile mortar, cut with small scissors, and ground thoroughly for 5 minutes, isotonic sodium chloride solution being added to obtain cell suspensions of 20% concentration. The suspensions were cleared from larger tissue pieces by passing them through a sterile voile cloth filter; the resulting cell suspension was used for inoculation of infant mice in Exp. No. 1126. In experiments 1100, 1101 and 1106, the leukemic cell suspensions, obtained in the manner described above, were centrifuged at 3,000 RPM for 15 minutes in a refrigerated PR-1 International Centrifuge at 0°C; the supernatant was removed carefully, placed in a new test tube, and again centrifugated for additional 15 minutes under identical conditions (3,000 RPM at 0°C). The second supernatant was then carefully removed with a tuberculin syringe, and used immediately for inoculation.

Embryo-cell suspension. A 4-month-old, healthy, pregnant female mouse No. 476 of the Ak inbred line was sacrificed, and 6 embryos were removed aseptically from the uteri. Over 70% of females of our colony of Ak mice developed spontaneously leukemia after they reached 7 months of age(1,2). The Ak female No. 476 was in good health at the time the embryos were removed from her uteri, because she was then only 4 months old. Her mother, Ak female No. 417 was also sacrificed, when in good health, when 5 months old, for experimental purposes. The grandmother, however, as well as great-grandmother, and several other ancestors on both her maternal and paternal sides had developed, and died from, spontaneous leukemia. The embryos thus obtained were cut with small scissors, and then ground thoroughly in a sterile mortar, isotonic sodium chloride solution being added to obtain a cell suspension of approximately 20% concentration. This cell suspension was then passed through a sterile voile cloth filter, tested for bacterial sterility, and used immediately for inoculation.

Experimental procedure. Females of the C3H (f) line were mated to their brothers in individual wooden cages; when they became pregnant, they have been watched at frequent intervals, and the birth of each litter was promptly recorded. In experiments reported in this study only litters less than 12 hours old were used for inoculation. Older suckling mice were also inoculated, but they will be reported separately(3). A tuberculin syringe, and a 27 gauge needle were used for the injection. The suckling mice were returned immediately after inoculation to their mothers. After they have reached 21 days of age, these mice were weaned, marked individually, and placed in separate cages. At the age of 2 to 3 months, the females were

1. Gross, L., *Cancer,* 1950, v3, 1073.

2. Cole, R. K., and Furth, J., *Cancer Research,* 1941, v1, 957.

3. Gross, L. Experiments to be published.

permitted to have one litter, having been mated to one of their respective brothers. After the litters had been weaned, these mice were separated according to sex, and were from then on kept under observation.

Results. Inoculation of newborn C3H (f) mice with leukemic cell suspensions. It was observed in our previous experiments that newborn mice of the C3H line are highly susceptible to inoculation with Ak leukemic cell suspensions. Of the 54 one-day-old infant mice inoculated, all developed acute leukemia in from 10 to 12 days(1). *Exp. 1126.* When, however, 5 one-day-old mice of the C3H line were inoculated subcutaneously with the Ak leukemic cell suspensions (0.1 cc each), only 4 of them developed acute leukemia within 12 days; the fifth infant mouse, a female, remained in good health; yet, after 7 months, this mouse developed generalized lymphatic leukemia. No leukemic infiltration was found under the skin, at the site of the initial subcutaneous inoculation.

Inoculation of newborn C3H (f) mice with Ak leukemic centrifugated extracts. Since the inoculation of newborn infant mice of the C3H line with Ak leukemic cell suspensions resulted in most instances in prompt development of acute leukemia, centrifugated leukemic extracts were used in experiments 1100, 1101, and 1106. These centrifugated leukemic extracts were injected subcutaneously, and/or intraperitoneally, into infant mice of the C3H (f) line. The inoculations were made within 12 hours after the birth of these animals. Of the 14 mice inoculated in 3 experiments, 7 developed generalized leukemia in from 8 to 11 months after the inoculation, as follows: *Exp. 1100.* Infant mice (1 female and 4 males) born on Jan. 9, 1950 to a C3H (f) female, were inoculated subcutaneously (each 0.1 cc) with Ak leukemic centrifugated extract, prepared from a transplanted Ak leukemia. Fourteen days later, a similar Ak leukemic centrifugated extract was freshly prepared from the same strain of transplanted leukemia, and the infant mice were reinoculated, this time intraperitoneally (0.1 cc each). These animals were weaned on February 3, 1950, and marked individually (female No. 22, males No. 293, No. 294, No. 295 and No. 296). All 5 mice remained in good health until October 1950. On October 13, 1950, 2 of these 5 mice, namely female No. 22, and male No. 295, were found to have generalized leukemia undistinguishable from "spontaneous" leukemia so frequently observed in middle aged mice of the Ak line. Aprroximately 2 months later, male No. 296 also developed generalized leukemia. None of these 3 leukemic mice had any local new growth at the site of the initial subcutaneous inoculation. *Exp. 1101.* A centrifugated extract was prepared from leukemic organs of a mouse of the Ak line that developed leukemia spontaneously. This extract was injected subcutaneously (0.1 cc each) into 4 infant mice (1 female and 3 males) of the C3H (f) line born less than 12 hours prior to inoculation on January 10, 1950. These infant mice were weaned on January 31, 1950 and marked individually (female No. 15 and males No. 286, No. 287 and No. 288). All 4 mice remained in good health until September, when male No. 287 developed generalized leukemia and died within a few days (September 7, 1950); there was no local infiltration at the site of the initial inoculation. On October 16, 1950, female No. 15 was found to have enlarged lymph nodes, and a few days later her spleen also became palpable. Blood count (from tail) revealed WBC 40,000 with 75% lymphocytes. The mouse was sacrificed, and a typical picture of generalized leukemia was found, with no evidence, however, of any local growth at the site of the initial subcutaneous inoculation. *Exp. 1106.* On January 16, 1950, a centrifugated leukemic extract was prepared in the usual manner from a transplanted Ak leukemia, and injected subcutaneously (0.1 cc each) into 8 infant mice of the C3H (f) line, less than 12 hours old. Of these 8 infant mice, 3 died within 2 weeks following inoculation. The 5 mice that survived, were weaned at 21 days of age, and given individual numbers (female No. 93, and males No. 63, No. 64, No. 65 and No. 66); they remained in good health until the first week in November, 1950; at that time male No. 63 developed generalized leukemia without any local growth at the site of the initial, subcutaneous

inoculation; a few days later, male No. 65 also was found to have developed leukemia.

Results of inoculation of Ak-embryos cell suspensions into infant mice of the C3H (f) line. Newborn infant mice of the C3H (f) line were inoculated subcutaneously, and also intraperitoneally, with cell suspensions prepared from Ak embryos. Suckling infant mice varying in age from those a few hours, to those several days old, were used for the inoculations. In this report, however, only 2 experiments are reported in which the infant mice were inoculated when they were less than 12 hours old; all the other experiments, dealing with mice inoculated at a later age, will be reported separately(3), because the results of all these other experiments have been thus far negative. *Exp. 1154.* On February 24, 1950, a cell suspension prepared from Ak embryos was injected subcutaneously, and also intraperitoneally (0.1 cc each), into 6 infant mice of the C3H (f) line, less than 12 hours old. Only 2 of these 6 infant mice survived the inoculation; the remaining 4 died after a few days. Of the 2 mice that survived, one (male No. 341) developed generalized leukemia at 8½ months of age, with no sign of any local growth at the site of the initial subcutaneous inoculation. *Exp. 1155.* On the same day (February 24, 1950) the Ak-embryo-cell suspension was injected subcutaneously and intraperitoneally (0.1 cc each) into 6 infant mice, less than 12 hours old, of the C3H (f) inbred line. Of these, 1 died within a week, and another was killed accidentally at the age of 3 months. Of the 4 mice that survived (females No. 118, No. 119 and No. 120, and male No. 96), one female (No. 118) developed generalized leukemia at 8½ months of age; her blood count, taken from tail, revealed 260.000 WBC with 65% of lymphocytes; there was no evidence of any local growth at the site of the initial subcutaneous inoculation. At the same time, male No. 96 also developed generalized lymphatic leukemia; in this animal again there was no evidence of any local growth at the site of the subcutaneous inoculation. One month later, female No. 120 was also found to have developed generalized leukemia.

Cell-transplantation of leukemia that developed spontaneously in C3H (f) mice. In an effort to bring some clarification of the nature of leukemia that developed in mice of the C3H (f) line reported in Experiments 1100 and 1101 of this study, an attempt was made to transplant this leukemia, by cell-transfer, into adult mice of the same inbred line. Accordingly, cell suspensions of 20% concentration, were prepared from C3H (f) females No. 22, Exp. 1100, and No. 15, Exp. 1101, and inoculated (0.1 cc each) intraperitoneally into 8 adult mice of the C3H line; as a result, all inoculated animals developed acute leukemia within 2 weeks. The results of both experiments suggest that females No. 22 and No. 15 developed "spontaneous" leukemia, since in both instances leukemic cells prepared from the donor animals were readily transplantable to adult mice of the same inbred line. Had these 2 mice (females No. 15 and No. 22) developed a "transplantable" Ak leukemia, as a delayed result of an inoculation, in their infancy, of Ak leukemic cells, a subsequent attempt to transplant the leukemic cell suspension from either mouse No. 22, or No. 15 to adult mice of the same C3H (f) line would have most likely not succeeded, unless massive doses had been employed(1,3).

Discussion. Experiments reported in this paper suggest that "spontaneous" leukemia can be prompted to develop in middle aged mice of a line known to be essentially free from this disease, by inoculating such mice, not later than 12 hours after their birth, with leukemic extracts prepared from either spontaneous, or transplanted mouse leukemia, or, in some instances at least, with embryo-cell suspensions prepared from apparently healthy mouse-embryos of a leukemic inbred line.

One could, perhaps, assume that leukemia developing in such middle aged animals is not "spontaneous," but may be a delayed result of the implantation, in infancy, of some leukemic cells that might have been present in the centrifugated extracts. The development of a "transplanted" leukemia, however, would rather be expected to occur much more promptly, within 2 to 3 weeks, usually(1);

another reason for doubt would be the fact that following subcutaneous inoculation of the centrifugated leukemic extracts, generalized leukemia developed in these hosts after middle age, without, however, any local new growth at the site of the initial subcutaneous inoculation. Finally, the successful results of the transplantation of leukemic cells from our C3H (f) mice No. 22 and No. 15, into adult mice of the same inbred line, are consistent with the assumption that females No. 22 and No. 15 had developed "spontaneous" leukemia, although it must be stated that in some instances, at least, Ak leukemic cells may also reproduce acute leukemia when inoculated, in large doses, into adult mice of the C3H line(1,4).

The development of leukemia in 4 out of 6 middle aged mice, that had been inoculated, within 12 hours after birth, with embryo-cell suspensions prepared from mouse embryos of a leukemic inbred line, is also quite interesting. No possibility of a leukemic cell implantation needs in these instances to be considered, since the cell suspension was in this experiment prepared from perfectly healthy embryos that had been removed aseptically from a healthy, young female mouse of a leukemic inbred line. The donor's mother had also been sacrificed in good health, but her grandmother, as well as great-grandmother, etc., died from leukemia. One could, perhaps, wonder whether the mice reported in this study developed leukemia by some obscure process of "mutation." One has, however, to consider the fact that in this particular inbred line leukemia develops in untreated animals only exceptionally, if at all. In our laboratory we have observed over 1100 mice of this foster nursed C3H subline, through 16 successive generations, without having found a single case of spontaneous leukemia among the untreated animals.

It would appear more logical to assume that these mice developed leukemia, in middle age, as a direct result of inoculation, in their early infancy, with a transmissible, leukemic agent. It would then be necessary to consider that this agent was present in the leukemic cells as well as in centrifugated leukemic extracts, and also in the Ak-embryo cell suspension; the leukemic agent was inoculated, into the newborn mice, in its inactive form; it remained inactive through the early adult life of its carrier-hosts; when the infected hosts, however, reached 8 to 11 months of age, the agent became, in some of them, at least, activated, causing the development of leukemia. Such a working hypothesis(5,6) may be new for mouse leukemia, but it has already been demonstrated to be true for chicken lymphomatosis(7,8). In principle at least, such a leukemia would be similar to mammary carcinoma which develops in middle aged female mice as a result of the infection of these animals, through mothers' milk(9), or by experimental inoculation, in their early infancy, with the mouse mammary carcinoma agent (10,11). The term "activated" would be, perhaps, more accurate than the term "spontaneous," since it is reasonable to assume that an activation of a hitherto latent agent, may be responsible for the "spontaneous" development of these neoplasms(5,6).

There are, however, important differences between the as yet hypothetical transmission of a mouse-leukemia agent, and the already established facts concerning the transmission of the mouse mammary carcinoma. Thus, the mouse mammary carcinoma agent is transmitted from mothers to their offspring through milk(9,10); in the case of mouse leukemia, however, it appears that the milk of nursing mothers is not responsible for the transmission of this disease from one generation to another(12-14); if such a transfer occurs at

4. Furth, J., Boon, M. A., and Kaliss, N., *Cancer Research,* 1944, v4, 1.

5. Gross, L., *Surg., Gynec., and Obst.,* 1949, v88, 259.

6. Gross, L., *Cancer,* 1951, in press.

7. Cottral, G. E., Proc. 53rd Ann. Meet. U.S. Livestock San. Assn., 1950, 183.

8. U. S. Dept. Agr. Tenth Ann. Rep. of the Reg. Poultry Res. Lab., E. Lansing, Mich., July 1, 1949 to June 30, 1950.

9. Bittner, J. J., *Science,* 1936, v84, 162.

10. Bittner, J. J. A.A.A. Research Conf. on Cancer, 1944, pp. 63-96.

11. Andervont, H. B., and Bryan, W. R., *J. Nat. Cancer Inst.,* 1944, v5, 143.

all, therefore, it would have to be explained by a direct transmission of the leukemic agent through the germinal cells, *i.e.* through the embryo, as in the case of chicken lymphomatosis(7,8). Another important point of difference between the agent of mammary carcinoma and that of mouse leukemia would refer to the time-limit of susceptibility of the hosts to experimental infection with these respective agents. When the experiments reported in this study were planned, we have anticipated that the susceptibility of mice of the C3H line to inoculation with the hypothetical leukemic agent may well be limited to the first days, or perhaps even weeks, of their lives; this assumption was based on certain theoretical considerations, mainly, however, on the fact that the susceptibility of mice to the experimental inoculation with the mammary carcinoma agent is for all practical purposes limited to their first 3 or at the utmost 4 weeks of life(10,15,16); similarly, the susceptibility of chickens to experimental inoculation with lymphomatosis has been found to be limited to their first few days of life(17). For these reasons, young, suckling infant mice were used for inoculations. Because, however, the older suckling mice are much more resistant to handling and to injections, the majority of those used for our experiments were 2 to 7 days old at the time of inoculation. Only very few newborn infant mice, less than 12 hours old, were used for inoculations with the leukemic extracts and, curiously, these were the ones that proved to be susceptible. Although more than 80 infant mice of the C3H (f) line were inoculated with leukemic extracts when they were more than 2 days old, they have all remained in good health up to the time of this writing(3). Similarly, only very few infant mice less than 12 hours old were used for inoculation with the Ak-embryo cell suspensions, and only some of those proved to be susceptible; when, however, older infant mice were inoculated, at ages varying from 2 to 7 days (25 females and 23 males)(3), none has developed leukemia up to the time of this writing, although more than one year has already passed since most of these experiments have been performed. Thus, the susceptibility of mice to experimental inoculation with the leukemic agent may well be limited to the very first hours of their lives.

Summary. 1. Seven of 14 C3H mice inoculated when less than 12 hours old with Ak leukemic extracts developed leukemia at 8 to 11 months of age. 2. Four of 6 C3H mice inoculated, when less than 12 hours old, with cell suspensions prepared from Ak embryos, developed leukemia at 8½ months of age. 3. No leukemia resulted when mice more than 12 hours old were used for inoculation. 4. These experiments suggest that spontaneous mouse leukemia may be caused by an agent, which is transmitted from one generation to another, like that of chicken lymphomatosis.

Received November 14, 1950. P.S.E.B.M., 1951, v76.

12. MacDowell, E. C., and Richter, M. N., *Arch. Path.*, 1935, v20, 709.

13. Barnes, W. A., and Cole, R. K., *Cancer Research*, 1941, v1, 99.

14. Furth, J., Cole, R. K., and Boon, M. C., *Cancer Research*, 1942, v2, 280.

15. Andervont, H. B., *J. Nat. Cancer Inst.*, 1945, v5, 397.

16. Dmochowski, L., *Brit. J. Exp. Path.*, 1945, v26, 192.

17. Waters, N. F., and Bywaters, J. H., *Poultry Sci.*, 1949, v28, 254.