visionally designated as Substance A. Further studies concerning enzymatic formation as well as purification and characterization of Substance A will be published.

1. Hamberg, U., Rocha e Silva, M., *Arch. int. Pharmacodyn.*, 1957, v110, 222.

2. Prado, J. L., Monier, R., Prado, E. S., Fromageot, C., *Biochem. et Biophys. Acta.*, 1956, v22, 87.

3. Hilton, S. M., Lewis, G. P., *J. Physiol.*, 1956, v134, 471.

4. Bernfeld, P. in Colowick S. P., Kaplan, N. V., *Methods in Enzymology*, vI, Academic Press, 1955, 149.

Received October 14, 1958. P.S.E.B.M., 1959, v100.

Serial Cell-Free Passage of a Radiation-Activated Mouse Leukemia Agent.* (24538)

LUDWIK GROSS (With technical assistance of Eleanor R. Mada, Yolande Dreyfuss and Lorraine A. Moore)

Cancer Research Unit, Vet. Admin. Hospital, Bronx, N. Y. City

Mice of the C3H inbred line do not usually develop "spontaneous" leukemia. Although the incidence varies in different laboratories, in our colony of C3H or C3H(f) mice (both of Bittner substrain) the incidence during past 10 years has not exceeded 0.5%(1). However, fractionated total body irradiation (150 r, 4 to 5 times, at weekly intervals) resulted in development of leukemia in over 50% of C3H mice after a latency period of approximately 6 to 7 months. Leukemia thus induced could then be transmitted, by filtrates, into newborn C3H mice; incidence was significant (11%), but not too high, and many extracts were inactive on inoculation tests (2). In our previous studies, some filtrates prepared from spontaneous Ak or C58 leukemias were also inactive on inoculation tests; however, selecting a potent filtrate and passing it serially through newborn mice, resulted eventually in development of a highly potent "passage A" leukemic virus inducing up to 90% leukemia after a latency of 3 to 4 months following inoculation into newborn or suckling C3H mice(3,1). Faced now with an apparently similar situation, we selected one of the more potent extracts among those prepared from radiation-induced C3H leukemias, and passed it serially through newborn C3H mice.

Materials and methods. All mice used were C3H or foster nursed C3H(f) mice, both of the Bittner subline. *Origin of leukemic virus strain.* The donor serving for preparation of the initial filtrate was a C3H female which, at age of 1½ months received a series of total body x-ray irradiation, 150 r each at weekly intervals, for 4 consecutive weeks. Five months after last irradiation, this mouse developed a very large thymic lymphosarcoma, and was then used as donor for preparation of the initial filtrate. *Preparation of filtrates for inoculation.* The leukemic donors were sacrificed by ether inhalation, and, without delay, parts of thymic and mesenteric tumors, peripheral lymph nodes, livers and spleens, were removed aseptically, weighed, and ground by hand in mortar with chilled, sterile physiological saline added, to obtain cell suspensions of 20% concentration. After centrifugation at 3,000 rpm (1,400 x g) for 15 minutes, the supernate was removed and again centrifuged at 9,500 rpm (7,000 x g) for 5 minutes. The second supernate (10 to 12 ml) was mixed with 0.5 ml of 1:2000 dilution of fresh broth culture of *E. coli*, and passed through Selas, porosity 02, porcelain filter candles, under vacuum pressure of approximately 20 mm mercury. All resulting filtrates were bacteriologically sterile, as evidenced by inoculating tryptose phosphate broth incubated for 24 hours; it was reasonable to assume, therefore, that no cells passed through filter candles. Kept at 0°C, most extracts

* Aided, in part, by grants from Damon Runyon Memorial Fund, and Am. Cancer Soc.

were used within a few hours, none later than after 24 hours. *Inoculation of filtrates.* Newborn, less than 16 hours old, or suckling 1 to 5 days old C3H, or C3H(f) mice, were inoculated subcutaneously or intraperintoneally. The sexes were separated at weaning time. Mice that died when less than 6 weeks old, were not included in the tabulation.

Results. Serial cell-free passage of leukemic agent from host to host. Only a cell-free passage, by means of filtrate, consisting of successful transmission of leukemic agent from host to host, was considered a consecutive "passage" and given a passage number. As in previous studies, this procedure was adopted, because it appeared questionable whether cell-transfer would either increase, or only sustain the infective potency of the leukemic agent. The passage agent was designated by the letter "X".

The *first passage* filtrate, prepared from a C3H female in which leukemia was induced by total body x-ray irradiation, was inoculated intraperitoneally into 2 C3H litters consisting of 8 mice less than 15 hours old. As a result 3 mice developed generalized lymphatic leukemia at 6½, 10½ and 11½ months respectively. The remaining mice died without signs of leukemia or tumors at 14½ months of age.

Second passage. A filtrate was prepared from one of the leukemic donors from the preceding passage, and inoculated subcutaneously into a litter of C3H mice consisting of 7 mice less than 16 hours old. Six mice developed generalized lymphatic leukemia at ages varying from 5 to 13 months.

Third passage. Two filtrates were prepared from 2 leukemic donors from the preceding passage, and inoculated into 4 litters varying in age from 2 hours to 2½ days. Of 16 mice, 13 thus far, (81%) developed generalized leukemia when 4 to 8 months old, and 3 are still alive and well at 8 months of age.

Fourth passage. Two filtrates were prepared from leukemic donors of the preceding passage, and inoculated into 9 mice varying in ages from 4 hours to 3 days. Four of these, thus far, developed generalized leukemia at 3 and 4 months respectively. The remaining 5 are still well at 4½ months of age.

Morphology of passage X filtrate induced leukemia. Mice which developed leukemia as a result of inoculation of passage X filtrates presented typical picture of generalized lymphatic leukemia, with enlargement of peripheral lymph nodes, large thymic and often also mesenteric tumors, enlarged spleens and livers. Most livers examined microscopically, showed typical lymphocytic infiltration around the large vessels. Peripheral (tail) blood counts were made on 11 leukemic mice. The number of white cells varied from 5,850 to 44,750/mm^3 (average 21,636 as compared with 10,355 in normal C3H mouse). Almost all leukemic blood smears showed presence of lymphoblasts (2%), and smudge cells, and most of them also nucleated red cells in peripheral blood; the dominant white cell was the lymphocyte, many of them showing atypical forms. All leukemic mice showed moderate to marked anemia (average 9.6 g Hb/100 ml as compared with 14.7 in the normal C3H mouse).

Controls. In a control group, 186 newborn C3H mice were inoculated with normal C3H organ extracts, and only 1 (0.5%) developed leukemia at 17 months of age, but 14 (7.5%) developed parotid gland tumors at 4.5 months average age(2).

One or two leukemic viruses? We were confronted, therefore, with the fact that in C3H mice, leukemia could be induced by a) inoculation of passage A(3) leukemic virus which originated from spontaneous Ak leukemia and was then passed serially through newborn C3H mice, or b) inoculation of a filtrate designated "passage X," which originated from radiation induced C3H leukemia. Incidence of induced leukemia was higher and the latency period shorter, when passage A filtrates were inoculated. Since, however, morphological differences between these 2 groups of leukemia were not sufficiently consistent to permit a basis for distinction, the question remained open whether we were not faced with the same disease, induced by the same virus, harvested from different sources. As a working hypothesis it was possible to assume that we were dealing with 2 distinct viruses; such

TABLE I. Results of Neutralization* *In Vitro* of Passage A Leukemic Virus with Inactivated (56°C ½ Hr) Guinea Pig and Rabbit Immune and Normal Serum.

	Pass. A leuk. immune serum		X-ray induced leuk. immune serum		Normal serum		Controls, leuk. fil.†	
	No. of mice inoc.	Leuk. inc., %	No. of mice inoc.	Leuk. inc., %	No. of mice inoc.	Leuk. inc., %	No. of mice inoc.	Leuk. inc., %
Rabbit serum	25	8	26	23	14	71	32	74
Guinea pig serum	44	34	47	85	17	41	54	78

* 20% passage A leukemic filtrate mixed 1:1 with undiluted serum, incubated at room temp. (22°C) for 30 to 60 min., then at 0° from 2 to 20 hr. All inoculations subcut. (138) or intraper. (121).

† Mixed 1:1 with physiol. saline.

All inoculated mice were of C3H or C3H(f) line (Bittner substrain); avg age at inoculation, 3 days. 22 mice died without signs of leukemia at avg age 9 mo. 93 mice surviving and well at present, avg age 11 mo.

Avg age leukemia developed: 4 mo in the controls and normal serum group; 4.7 mo in both immune serum groups.

an assumption, however, remained to be proven.

Attempt to neutralize mouse leukemia virus by a specific serum. A group of young, adult rabbits and guinea pigs, received at 7 to 10 days intervals, 6 to 8 intraperitoneal injections of leukemic filtrates prepared from passage A leukemic C3H donors. Another group of rabbits and guinea pigs received simultaneously a similar number of intraperitoneal injections of filtrates prepared from passage X leukemia, or of filtrates prepared from C3H donors in which leukemia was induced by total body x-ray irradiation. Both groups of rabbits and guinea pigs were bled 7 to 10 days after last injection. As a control, normal serum was obtained from untreated rabbits and guinea pigs. Serum from rabbits, or guinea pigs, in each group, was pooled, and used either fresh, or after inactivation at 56°C for 30 minutes. The undiluted serum from each group was then mixed 1:1 with a freshly prepared 20% passage A leukemic filtrate, incubated at room temperature (22°C) for 30 to 60 minutes, then for additional 2 to 20 hours at 0°C, and inoculated into suckling, 1 to 7 day old, C3H mice.

Fresh immune guinea pig passage A, or passage X, serum neutralized the passage A leukemic agent. Of 36 mice inoculated with leukemic filtrate mixed with fresh passage A serum, none developed leukemia; of 33 mice inoculated with leukemic filtrate mixed with fresh passage X serum, only 7 developed leukemia at 5 months of age. This neutralizing action of both immune sera was not specific however, since normal, fresh guinea pig serum had a similar neutralizing effect: thus, of 17 mice inoculated with leukemic filtrate mixed with normal guinea pig serum, one developed leukemia; (all 78 surviving mice are now in good health at 7.5 months of age). In a simultaneous control group, 18 mice were inoculated with leukemic filtrate mixed 1:1 with physiological saline solution, and all developed leukemia at average age of 3 months.

The results were different, when inactivated (56°C for 30 minutes) serum was used for neutralization tests (Table I). The passage A immune rabbit serum had a marked neutralizing effect on passage A leukemic agent, only 8% of inoculated mice developing leukemia, as compared with 74% in the control group, and 71% or 23% respectively in groups where normal or passage X rabbit sera were used.

Discussion. Experiments here reported suggest that normal, healthy mice of the low-leukemic C3H line, may carry a masked, usually non-pathogenic, leukemic agent. Triggered by ionizing radiation, this agent may become pathogenic, causing leukemia in its carrier host. Such an agent may then be transmitted, by filtrates, to other C3H mice, provided that it is inoculated into newborn hosts. The potency of filtrates prepared from different C3H donors with radiation-induced leukemia may vary considerably. Of 18 filtrates prepared from individual C3H donors with radiation induced leukemia, and inoculated into newborn C3H mice, 11 proved to be active on inoculation tests(2). It was ap-

parent therefore that a transmissible, filterable leukemogenic agent could be activated in at least 11 different C3H mice by total body x-ray irradiation. Selecting a potent extract, the agent could be passed serially through 4 consecutive cell-free passages of newborn C3H hosts.

Whether the filterable leukemic agent, designated "passage X" is distinct from leukemic virus designated passage A which originated from spontaneous Ak leukemia and which has been also passed serially in C3H mice, remains to be determined. Since the concentration of the virus in extracts used is not known, it is not possible to differentiate virus A from virus X on the basis of serum neutralization tests here reported. Thus, the assumption that the leukemic viruses A and X are distinct, although possibly related, remains a working hypothesis, and still requires experimental confirmation.

Summary. 1. A filterable leukemic agent designated "virus X" recovered from a C3H female in which leukemia was induced by x-ray irradiation, was passed serially through 4 consecutive cell-free inoculations of suckling C3H mice. 2. Of 40 mice inoculated, 26 thus far developed leukemia (65%) at ages varying from 3 to 11 months. 3. Inactivated (56°C ½ hr) rabbit and guinea pig serum, prepared with agent X filtrates, only partially neutralized passage A leukemic agent; passage A immune rabbit serum had a distinct, though not complete neutralization effect on the passage A agent.

1. Gross, L., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 300.

2. ———, *Acta Haemat.*, 1958, v19, 353.

3. ———, PROC. SOC. EXP. BIOL. AND MED., 1957, v94, 767.

Received October 20, 1958. P.S.E.B.M., 1959, v100.

Site of Reaction of Wax Bean Hemagglutinin with Rabbit Erythrocytes.* (24539)

ROBERT L. NORTHROP† AND IRVIN E. LIENER (Introduced by M. O. Schultze)
Dept. of Agricultural Biochemistry, University of Minnesota, St. Paul

Many plant seeds contain substances which agglutinate animal erythrocytes(1). Experiments reported here reveal several features of rabbit erythrocyte agglutination common to wax bean hemagglutinin (WBH) and to influenza and Newcastle Disease viruses. This suggests that both may act on the same site of the red cell membrane.

Materials and methods. Preparation of WBH. A partially purified preparation was obtained as follows. To 50 g of finely pulverized stringless wax beans‡ (*Phaseolus vulgaris*) was added 500 ml distilled water, and pH of suspension was adqusted to pH 7. Insoluble material was removed from suspension by centrifugation, and pH of supernatant was acidified to pH 4.6. Material precipitating out at this pH was discarded, and the supernatant fully saturated with $(NH_4)_2SO_4$. The precipitated protein was dialyzed against distilled water and finally lyophilized. The *receptor destroying enzyme* (RDE) was prepared from *Clostridium perfringens* as described by Popenoe and Drew(2). *Virus-treated blood cells* were prepared by adding 10 ml of chorioallantoic fluid [obtained from 10-day-old embryonated eggs inoculated with influenza (PR 8) or Newcastle Disease (NDV) virus§ and harvested after incubation at 37° for 48 hours] to 30 ml of 1.5% suspension of trypsinated rabbit erythrocytes(3). 0.9% NaCl replaced

* Paper No. 4023 Scientific Journal Series, Minn. Agric. Exp. Station. This work was supported by grant from Graduate School, Univ. of Minnesota.

† Present address: Med. School, Univ. of Wisconsin, Madison.

‡ Purchased from Farmer Seed and Nursery Co., Faribault, Minn.

§ Generously provided by Dr. J. T. Syverton, Dept. of Bacteriology, Univ. of Minnesota, Minneapolis.