albumin fraction predominates. Serum globulin increases with age, but serum albumin shows little change. Hypercholesterolemia is observed in 640-day-old rats.

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Neutralization *in vitro* of Mouse Leukemia Virus by Specific Immune Serum. Importance of Virus Titration.* (30200)

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After it was demonstrated that leukemia in mice is caused by a filterable and transmissible virus(1), and the experimental conditions were determined under which a leukemogenic virus could be isolated from spontaneous mouse leukemia, a large number of individual isolations of leukemogenic viruses in mice have been reported from tissues of mouse donors with spontaneous(2), or radiation-induced(3,4) leukemia, and also from transplanted mouse tumors(5). Most of these viruses induce in mice, and also in rats, essentially the same disease; they also have similar biological and physical properties. The question has been raised whether the many leukemogenic viruses isolated from leukemic mouse organs, and from transplanted mouse tumors during the preceding decade represent individually distinct viruses, or whether they represent isolations from different sources of the same mouse leukemia virus, or one of its close variants(2).

This study deals with an attempt to identify one of the relatively potent mouse leukemia virus strains recently isolated from non-leukemic mouse tissues. Following the

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method originally described by Graffi(5), Moloney(6,7) isolated a potent leukemogenic virus from a transplanted mouse sarcoma. This virus induces in mice, and also in rats, leukemia, usually lymphatic, in some instances also myeloid, and as tissue reactions lymphosarcomas, and less frequently also reticulum-cell sarcomas. The forms of leukemia and lymphomas induced with the Moloney strain are essentially indistinguishable from those induced with passage A virus, originally isolated from spontaneous mouse leukemia.

The purpose of this study was to determine whether there are antigenic differences between the passage A virus and the virus strain isolated by Moloney, and whether both could be neutralized, *in vitro,* by the same immune serum.

Results of experiments here reported suggest that neutralization of the virus *in vitro* by an immune serum will occur only under carefully performed quantitative conditions. When a filtrate containing an unknown, and possibly highly potent, concentration of the mouse leukemia virus is mixed with a heterologous immune serum, neutralization may not occur even though the serum contains specific antibodies. This difficulty is due to the low potency of the immune serum, and the relatively high titer of virus present in routinely prepared filtrates.

Materials and methods. Passage A virus and preparation of extracts. Serial passages 33 to 42 of the mouse leukemia virus, designated passage A, originally isolated from spontaneous Ak mouse leukemia and then passed serially by filtrate inoculation through newborn C3H mice(8), were employed. Fragments of pooled thymic tumors and spleens from several C3H(f) mice with passage A virus-induced leukemia were removed aseptically, weighed, cut with scissors, and ground in a mortar with physiological saline solution to obtain a cell suspension of 20% concentration; following centrifugation at 0°C at 3,000 r.p.m. for 15 minutes, then at 9,500 r.p.m. for 5 minutes, the second supernate was passed through Selas, porosity 02 filter candles, and the filtrate was either immediately used for inoculation, or stored in sealed ampoules at —70°C in an electric Revco Ultra Low Temperature cabinet, and then used within one month.

Rat-adapted passage A virus. In certain experiments (Table II), the filtrates were prepared from rats with passage A virus-induced leukemia(9). Seventh to 12th consecutive rat-to-rat passage of the virus was employed. The method of preparation of filtrates was the same as that employed for filtrates prepared from mouse donors.

Moloney strain. In October, 1962, we received from Dr. John B. Moloney, National Cancer Institute, several BALB/c mice inoculated with his virus strain. As soon as these mice developed leukemia, we prepared filtrates from their leukemic organs and passed this virus strain serially through suckling, less than 6 days old C3H(f) mice. The incidence of induced leukemia was practically 100% and the average latency approximately 3½ months. From C3H(f) donors with Moloney-strain-induced leukemia, filtrates were prepared in exactly the same manner as that employed for preparation of passage A filtrates.

Animals. All mice employed in this study were of the C3H(f) strain, Bittner subline, free from the mammary carcinoma agent by foster nursing, and raised in our laboratory by brother-to-sister mating. The incidence of spontaneous leukemia in these mice has been about 0.5%.

Preparation of immune rabbit sera. Young adult female rabbits, 2 in each group, were inoculated subcutaneously and intraperitoneally, with extracts described below. The extracts, in the form of filtrates, were either freshly prepared immediately prior to inoculation, or prepared, then frozen in sealed ampoules, and kept up to one month at —70°C, until used. One week after the last immunizing injection, the rabbits were bled by heart puncture. The blood was distributed in sterile tubes, and allowed to clot; the serum was then separated by centrifugation, and pooled from both animals that had received identical injections. The pooled serum was inactivated at 55°C for 30 minutes, distributed in glass ampoules, and stored at —20°C until

TABLE I. Titration of Passage A Virus* and of Moloney Strain in Suckling, <6-Days-Old, C3H(f) Mice.

Passage A virus filtrate					Moloney strain filtrate				
Virus fil. dil.	No. of mice inoc.†	No. dev. leuk.	Leuk. incid., %	Avg latency, mo	Virus fil. dil.	No. of mice inoc.†	No. dev. leuk.	Leuk. incid., %	Avg latency, mo
10^{-2}	17	16	94	2.8	10^{-2}	10	10	100	3.9
10^{-3}	41	39	95	3.3	10^{-3}	15	15	100	4.5
10^{-4}	32	29	91	3.8	10^{-4}	20	17	85	4.6
10^{-5}	54	30	56	4.7	10^{-5}	27	24	89	5.3
10^{-6}	14	2	14	8.8	10^{-6}	22	17	77	5.9

* Serial passage 33 to 42 in newborn mice. † 0.3 ml i.p. each.

TABLE II. Titration of Passage A Virus (Rat-Adapted)* in Suckling Rats and Mice.

Titration in suckling rats†					Titration in suckling mice‡				
Virus fil. dil.	No. of rats inoc.	No. dev. leuk.	Leuk. incid., %	Avg latency, mo	Virus fil. dil.	No. of mice inoc.	No. dev. leuk.	Leuk. incid., %	Avg latency, mo
10^{-1}	6	6	100	2.8	10^{-1}	6	6	100	3.1
10^{-2}	13	13	100	4.3	10^{-2}	14	14	100	4.9
10^{-3}	25	19	76	4.2	10^{-3}	17	15	88	4.8
10^{-4}	20	5	25	6.1	10^{-4}	23	19	83	4.5
10^{-5}	23	2	9	3	10^{-5}	15	11	73	5.2

* 7th to 12th serial rat-to-rat passage.
† <7-day-old Sprague-Dawley rats, inoculated 0.4 ml i.p. each.
‡ <6-day-old C3H(f) mice, inoculated 0.3 ml i.p. each.

used. List and details of preparation of sera employed follow.

Exp. No. 1, Table III. A) *Passage A (mouse) filtrate with adjuvant.* Two rabbits received 3 consecutive injections (5 ml each), at one-week intervals, of passage A virus filtrate of 20% concentration, harvested from mice, and mixed with equal quantity of complete adjuvant (Difco Bacto Adjuvant, complete, Freund). Subsequently, these rabbits received one additional injection of virus filtrate mixed with incomplete adjuvant (Difco Bacto Adjuvant, incomplete, Freund), followed after 2 weeks by a final booster of virus filtrate only. B) *Normal mouse organs with adjuvant.* Two rabbits received 3 consecutive injections (5 ml each), at one-week intervals, of filtrate of 20% concentration, prepared from normal mouse organs, such as thymus, liver, spleen, and lymph nodes, removed from young, healthy, non-injected C3H(f) mice. This was followed by inoculation of normal mouse organs filtrate mixed with complete adjuvant; after one month interval, a similar filtrate was injected, mixed with incomplete adjuvant. After 2 weeks, a final booster dose was injected of normal organs filtrate only.

Exp. No. 2, Table III. C) *Passage A (rat) with adjuvant.* Two rabbits received 4 consecutive injections (5 ml each), at one-week intervals, of passage A filtrate of 20% concentration, harvested from rats (rat-adapted virus), followed by injection of 5 ml of a similar virus filtrate mixed with complete adjuvant; this was followed 2 weeks later by injection of virus filtrate mixed with incomplete adjuvant, and subsequently by a final booster inoculation of filtrate only, without adjuvant. D) *Normal rat organs with adjuvant.* Two rabbits received (5 ml each) a filtrate of 20% concentration, prepared from normal organs, such as thymus, spleen, liver, peripheral lymph nodes, etc. removed from young, healthy, non-injected Sprague-Dawley rats. After one week the rabbits received the same filtrate mixed with complete adjuvant; this was followed 2 weeks later by injection of filtrate mixed with incomplete adjuvant. After 3 weeks, a final booster dose was injected consisting of normal rat organs filtrate only.

TABLE III. Neutralization *in vitro** of Passage A Mouse Leukemia Virus, and of Moloney Strain, by Immune Passage A Rabbit Serum.

Exp	Antigen employed for preparation of rabbit serum	Serum dil.	Passage A virus					Moloney strain				
			Virus fil. dil.	No. of mice inoc.†	No. dev. leuk.	Leuk. incid., %	Avg latency, mo	Virus fil. dil.	No. of mice inoc.†	No. dev. leuk.	Leuk. incid., %	Avg latency, mo
1	Passage A virus harvested from mice (with adj.)	undil.	10^{-3}	8	0	0	—	10^{-3}	11	1	9	8
	Normal mouse organs (with adj.)	"	10^{-3}	9	9	100	3.7	10^{-3}	13	13	100	5.5
	Saline controls		10^{-3}	6	6	100	3.4	10^{-3}	3	2	67	3
2	Passage A virus harvested from rats (with adj.)	"	10^{-3}	6	1	17	5	10^{-3}	14	13	93	5
	Normal rat organs (with adj.)	"	10^{-3}	8	8	100	3.1	10^{-3}	6	5	83	4.3
	Saline controls		10^{-3}	9	8	89	3.2	10^{-3}	7	7	100	4.7
3	Passage A virus harvested from mice & rats (no adj.)	1:2	10^{-2}	15	4	27	6	10^{-2}	21	20	95	5.2
	Normal rabbit serum	1:2	10^{-2}	8	8	100	2.9	10^{-2}	13	13	100	4.8
	Saline controls		10^{-2}	19	18	95	3	10^{-2}	16	16	100	4.1
4	Passage A virus harvested from mice & rats (no adj.)	1:2	2.5%	11	10	91	4.8	2.5%	14	14	100	4.7
	Normal rabbit serum	1:2	2.5%	8	8	100	3.1	2.5%	14	13	93	4.5
	Saline controls		2.5%	6	5	83	3.1	2.5%	5	5	100	4.8

* Virus filtrate mixed with equal amount of undiluted, or 1:2 dilution of serum, incubated at room temperature for 30 min (in Exp 1 also at 37°C for 25 min) then for 3½ to 4 hr at +4°C.

† Suckling, <6-day-old C3H(f) mice inoculated 0.3 ml i.p. each. All animals observed for 9 to 10 months for development of leukemia.

Exp. No. 3 and 4, Table III. E) *Passage A (mouse and rat) without adjuvant.* Two rabbits received up to 12 consecutive injections (5 ml each), at about one-week intervals, of virus filtrate of 10% to 20% concentration, harvested either from mice, or from rats, with virus-induced leukemia. No adjuvant was added to any of these filtrates. F) *Normal rabbit serum.* This was normal rabbit serum, collected from normal, non-injected, young, healthy rabbits.

Experimental. Preliminary titration of passage A virus in mice. Passage A mouse leukemia filtrate of 10% concentration (10^{-2}) was prepared in the usual manner; this initial filtrate (10^{-2}) was serially diluted. Each dilution was then tested by inoculating suckling, less than 6 days old, C3H(f) mice (0.3 ml i.p. each). It is evident from results (Table I) that 10^{-2} through 10^{-4} dilutions induced an incidence of leukemia exceeding 91%; this incidence dropped to 56% when 10^{-5} dilution was inoculated, and to only 14% when a 10^{-6} dilution was employed; the latency gradually increased from 2.8 months in the 10^{-2} group to 8.8 months in the 10^{-6} group.

Titration of the Moloney strain. When in a similar experiment a filtrate prepared from the Moloney strain was inoculated in serial dilutions into suckling, less than 6 days old, C3H(f) mice, the incidence of induced leukemia was 100% when 10^{-2} and 10^{-3} dilutions were inoculated, exceeded 85% when 10^{-4} and 10^{-5} dilutions were injected, and was still 77% when a 10^{-6} dilution was employed (Table I).

Titration of rat-adapted passage A virus, in suckling rats and mice. After some 7 to 12 consecutive rat-to-rat passages of the passage A virus in suckling rats, the virus harvested from leukemic rats was titrated in suckling rats and also in suckling mice. It is apparent from results summarized in Table II that the susceptibility of suckling rats to this virus was lower than that of suckling mice. The incidence of leukemia induced in rats with a 10^{-3} dilution of the filtrate was 76%, and dropped to 25% when 10^{-4} dilution was inoculated; only 2 out of 23 rats developed leukemia following inoculation of 10^{-5} dilution. On the other hand, when suckling mice were inoculated with the same filtrates, the incidence of leukemia was 88% and 83% when either 10^{-3} or 10^{-4} dilutions were inoculated respectively, and 73% when a 10^{-5} dilution was employed.

Serum neutralization tests. Virus filtrates containing either the passage A virus or the Moloney strain, were mixed with equal amounts of undiluted serum, or with a 1:2 dilution of serum. The mixtures were thoroughly stirred, left at room temperature for 30 minutes, (in Exp. No. 1 also for 25 minutes at +37°C), then for 3½ to 4 hours at +4°C. The virus-serum mixtures were then inoculated (0.3 ml i.p. each) into suckling C3H(f) mice less than 6 days old. The injected animals were then observed for development of leukemia. Four successive experiments were performed. Neutralization of both passage A virus and of the Moloney strain was obtained when 10^{-3} dilutions of the virus filtrates were mixed with equal amounts of undiluted serum obtained from rabbits that had received passage A mouse virus with adjuvant. There was no neutralization of either virus when in control experiments serum was employed from rabbits that had received normal mouse organ filtrates with adjuvant (Exp. 1, Table III).

An immune serum obtained from rabbits that had received injections of rat-adapted passage A virus with adjuvant was less potent (Exp. 2, Table III). Such a serum still neutralized the passage A virus filtrate, but was not sufficiently potent to neutralize the Moloney strain in 10^{-3} dilution. The Moloney strain filtrate had a substantially higher virus titer than the passage A virus filtrate (Table I). This could account for the results obtained in this experiment.

Serum from rabbits that had received injections of the passage A virus filtrate without the adjuvant had a still lower antibody content. Neutralization of the passage A filtrate with such a serum was only partial. There was no neutralization under such conditions of the Moloney strain (Exp. 3, Table III).

It was of interest that when a 2.5% dilution of the filtrate was employed, there was

essentially no neutralization of either passage A virus or of the Moloney strain by a 1:2 dilution of the immune serum (Exp. 4, Table III).

Discussion. It is necessary to differentiate between the various forms of a specific, although complex, syndrome designated by the term mouse leukemia, and another disease, induced by the Friend virus(10), which may belong to the broad group of "leukemias" in mice, but actually represents a different syndrome.

The term mouse leukemia here employed (2) refers to a limited variety of this disease observed in mice to develop spontaneously, or induced either by X-ray irradiation, carcinogenic chemicals, or hormones. The most frequently observed form is lymphatic, but may be also stem-cell or myeloid. The prominent feature is usually, but not always, a large thymic lymphosarcoma, frequently also a large spleen, and prominently enlarged lymph nodes, due to infiltration with leukemic cells. The term "leukemia" can be applied to this form of disease only in its broad and general meaning; it does not necessarily imply changes in peripheral blood morphology. Actually, the most commonly occurring form of leukemia in mice does not show leukemic manifestations in peripheral blood, and could be designated as a generalized lymphosarcoma. It appears that all these forms of leukemia and lymphomas can be induced in mice by a single virus; it was suggested(2) to designate this virus "mouse leukemia virus type A."

A different disease of the hematopoietic system, which has not been observed to develop spontaneously, can be induced in young adult mice of susceptible strains following inoculation of a virus isolated by Friend (10). This virus induces a progressive enlargement of spleen and liver with destruction of parenchymal cells, and cellular infiltration resembling that observed in leukemia. Significantly, the thymus is not involved, and neither are the peripheral or internal lymph nodes. There is a severe and rapidly progressing anemia; the peripheral blood contains many nucleated red cells, a very large number of "smudge cells," and also many cells of the myeloid series. The virus strain recently isolated by Rauscher (12) can induce not only the Friend disease, but also lymphatic leukemia similar to that resulting from inoculation of the passage A mouse leukemia virus. It is quite possible that the Rauscher strain is a mixture of the Friend virus, and of the "type A" mouse leukemia virus(11). It was observed recently (11) that inoculation of the Friend virus into either suckling or young adult (weanling) mice of strain C57/Brown may also result in development of lymphatic leukemia. It is possible therefore that the Friend virus filtrates contain not only the Friend virus, but also the "type A" mouse leukemia virus.

When the mouse leukemia virus was originally isolated from spontaneous Ak mouse leukemia(1), it had a relatively narrow host range. The pathogenic potency of this virus increased after passage through newborn mice(8); its host range became considerably broader(13). Moreover, it was realized that the form of induced disease depends to a considerable extent on the susceptibility of the host. Following inoculation of this virus into suckling mice of a variety of susceptible strains, as well as into rats, several forms of leukemia and lymphomas could be induced(14). The most frequently induced form was that which could be designated as aleukemic lymphatic leukemia, or a generalized lymphosarcoma; lymphatic leukemia involving peripheral blood, as well as stem-cell leukemia could also be induced; myeloid leukemia was induced frequently in mice of certain strains, such as BALB/c(13), and also in rats(9); following thymectomy, in mice of strain C3H, not only myeloid leukemia could be induced, but also certain less frequently observed forms, such as chloroleukemia(15). It became apparent that the virus originally isolated from spontaneous mouse leukemia designated "passage A" is capable of inducing in mice and rats a spectrum of all the usually observed forms of leukemia and lymphomas(2).

Many isolations have been reported of presumably distinct viruses causing mouse leukemia(2). That some of these viruses may induce predominantly lymphatic leukemia,

others a relatively high incidence of myelogenous leukemia, and still others reticulum-cell sarcomas, etc., does not necessarily imply that they represent different viral agents. The variety of forms induced depends to a considerable extent on the individual susceptibility of the host. We are therefore faced with the fact that the usual forms of mouse leukemia and lymphomas can be induced in mice and rats with several filterable mouse virus strains isolated from a variety of sources. These different virus strains have very similar, if not identical, pathogenic, biological and physical properties(2). To identify such viruses, serological tests may be necessary. An attempt was made in this study to use the serum neutralization test for identification of one of the most potent mouse leukemia virus strains recently isolated by Moloney(6) from a transplanted mouse sarcoma, in order to determine whether this strain represents a new and different mouse leukemia virus, or whether it represents isolation, from another source, of the same virus that had been previously harvested from spontaneous mouse leukemia(1,2,8).

Experiments here reported suggest clearly that under proper and quantitative experimental conditions, both the passage A, and the Moloney strain, can be neutralized *in vitro* by the same immune rabbit serum. The passage A virus and the Moloney strain have identical physical properties; both can be inactivated by heating to 50°C for 30 minutes, and are sensitive to ether(2); they are morphologically indistinguishable on electron microscopic examination(16,17); both have a similar host range, and both induce the same disease in mice and rats. Results of experiments reported here, suggesting that there are no antigenic differences between these two viruses, and that they both can be neutralized *in vitro* by the same immune serum, are consistent with the assumption that the passage A virus and the Moloney strain represent the same virus.

The only difference is that of the titer. Prepared under similar experimental conditions, the Moloney strain has a higher titer than the passage A virus filtrate. This is evident from Table I, which indicates that the Moloney strain filtrate still induced a considerable number of leukemias following inoculation of a 10^{-5}, and even a 10^{-6}, dilution. The Moloney strain was isolated from sarcoma S-37, which has been transplanted by cell graft from mice to mice for over 50 years; it is possible to speculate that a leukemic virus picked up somewhere in the course of the Sarcoma 37 cell transplantations, and transmitted serially as a "passenger virus," increased considerably in potency after many years of consecutive mouse-to-mouse transfers, even though it remained latent in its carrier hosts.

Summary. 1) Both passage A virus and Moloney strain were neutralized *in vitro* by the same specific serum obtained from rabbits immunized by repeated injections of passage A virus filtrates. 2) No neutralization of either passage A virus, or Moloney strain, occurred in control experiments in which the serum employed was obtained from rabbits that had received repeated injections of normal mouse organ filtrates. 3) Neutralization occurred only when 10^{-3} dilution of either virus filtrate was mixed with equal amount of undiluted immune serum. When higher concentration of virus filtrate, or a less potent serum, were employed, neutralization was less pronounced, and affected only passage A virus, or did not occur at all. 4) Injection into rabbits of passage A virus harvested from mice produced more potent serum than rat-adapted virus. Addition of adjuvant to virus filtrate injected into rabbits increased the potency of serum. 5) Prepared under identical experimental conditions, Moloney strain filtrate had a higher virus titer than passage A filtrate. This could explain why serum of lesser potency could still neutralize, in part, a 10^{-3} dilution of passage A filtrate, but did not neutralize a 10^{-3} dilution of Moloney strain filtrate. 6) Passage A virus filtrate induced leukemia in higher dilutions in mice than in rats. The mouse is considerably more susceptible to this virus than the rat.

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Hemorrhagic Disease in Rodents Caused by Chikungunya Virus. 1. Studies of Hemostasis. (30201)

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It has been shown that chikungunya virus (CHIK), isolated from a Thai child with mild hemorrhagic fever, produced a fatal hemorrhagic disease when inoculated into suckling rodents(1). Following a dose-related, asymptomatic incubation period of 2-5 days, the animals rapidly became ill, as manifested by sluggishness, poor feeding, cyanosis, decrease in skin temperature, cardiac enlargement and death. A significant proportion of animals develop grossly visible hemorrhages into the gastrointestinal tract and, to a lesser extent, into the bladder, joints and skin. Histologically, there are intraalveolar hemorrhages, focal skeletal muscle and fat pad necrosis and a diffuse enteropathy characterized by a decrease in goblet cells, thickening and blunting of villi in the small intestine, crypt formation, basophilia of the epithelial cytoplasm and marked congestion of the intestinal blood vessels in the lamina propria, submucosa and serosa.

The results of studies on the hemostatic abnormalities on these animals are presented here. Attempts were also made to modify the occurrence of hemorrhage by pretreating animals with various drugs.

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Materials and methods. Hosts. Litters of eight or nine 24-36-hour-old suckling mice, Walter Reed Bagg strain, or suckling Wistar rats were used. Age of suckling animals was determined by noting births at 6-hour intervals.

Virus. KLA-16 strain of CHIK was used; it produced a high percentage of hemorrhage. Mice were infected with 10-1000 LD_{50} of virus obtained after 4-6 mouse passages (sm 4, 5, 6). Rats were infected with virus which had been passaged 3 times in mice and once or twice in suckling rats (sm3r1 or sm3r2).

Observation of hemorrhage. Between 60-96 hours following intracerebral inoculation of 0.01 ml of virus, rodents were observed at 6-hour intervals. Such frequent observation was necessary to detect presence of sick animals before they were destroyed by mothers. Animals with grossly visible gastrointestinal hemorrhage were recorded and removed from the cage. Occurrence of hemorrhage was expressed as the number with gastrointestinal hemorrhage divided by the total infected and surviving on the second day following inoculation. In experiments in which length of survival was of importance, the animals with hemorrhage were not removed but observed