Again, agglutination in goat rbc closely paralleled sheep rbc agglutination except that the titer was higher with goat cells. Fifteen of these serums were tested by ox cell hemolysin and none reacted.

The last 3 serums at bottom of Table II showed a low titer reaction different from other negative ones. The titer of antibody in the presumptive test (1:14 with sheep rbc and 1:28 with goat rbc) was too low to be considered positive, but it was not removed by absorption with guinea pig kidney in 1 serum with sheep cells and in all 3 serums with goat cells. It was removed in each case by beef rbc antigen. Two of these serums were tested in the ox cell hemolysin test and failed to react.

Summary and conclusions. Sera from 61 patients showed similar reactions with both sheep and goat rbc in the differential agglutination test for infectious mononucleosis. Twenty-seven sera caused positive differential agglutination with both sheep and goat rbc and reacted in the ox cell hemolysin test. No other sera reacted positively in any of the tests. Goat cells reacted with higher dilution of serum than sheep cells with both patient serums and with 100 normal serums. A positive reaction in goat rbc was of approximately 4-fold higher titer than with sheep rbc. Within limits of these investigations it is concluded that the differential agglutination test with goat rbc and the ox cell hemolysin test gives the same laboratory information for diagnosis of infectious mononucleosis as is obtained from the sheep rbc agglutination test.

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Agglutinating Action of Heat-Inactivated Passage A Mouse Leukemia Filtrates on Mouse Red Blood Cells.* (24850)

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Since a potent mouse leukemia virus (Gross) has been developed by serial cell-free passage through newborn mice of an Ak-leukemia-derived agent(1,2), an attempt was made to determine whether leukemic filtrates containing this agent would agglutinate, or hemolyze, mouse red blood cells (rbc) *in vitro*. In preliminary tests, heated (60°C ½ hr) filtrates were also used, as controls. The surprising observation was made that fresh filtrates were with rare exceptions essentially inactive, whereas heated filtrates had consistently a distinct agglutinating effect on mouse erythrocytes. Experiments were therefore carried out to determine some of the conditions related to agglutinating action of mouse leukemia filtrates on mouse rbc *in vitro*.

Methods. Filtered (Selas 02) extracts of 20% concentration were prepared in usual manner(1-3) from C3H donors with primary, passage A(1,2) virus-induced leukemia. Part of extract was placed in ice-water at 0°C, and used within a few hours for the test. Another part of extract was heated at 55°C for ½ hour. In a few experiments, extracts were also heated to temperatures varying 40° to 60° for ½ hour. Similar extracts were prepared from spontaneous Ak mouse leukemias, from several spontaneous C3H mouse mammary carcinomas, from x-ray induced C3H leukemias, from normal organs (spleen, liver, heart, lungs, kidneys, testicles), and embryos, removed from young healthy C3H mice. *Red blood cells* were obtained by heart puncture,

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TABLE I. Agglutination of Mouse RBC by Heated (56°C ½ Hr) Filtrates from (a) Passage A Leukemic, (b) Spontaneous Ak Leukemic, and (c) Normal C3H, Donors.

Extracts from donors	No. of extracts tested	No. of extracts agglutinating in dilutions						
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Passage A leuk.	18	18	17	16	13	6	2	0
Spontaneous Ak leuk.	10	7	5	3	2	1	0	0
Normal C3H	9	5	2	1?	0	0	0	0

performed with light ether anesthesia, from young healthy C3H or C3H(f) mice. In a few experiments, red blood cells were also obtained from young, healthy Ak mice, and also from guinea pigs, and rabbits. Suspended first in physiological saline solution mixed with sodium citrate to prevent coagulation, they were washed twice briefly, with, and then resuspended (1%) in, chilled sterile physiological saline solution. Erythrocytes were used within a few hours after removal from animals' blood circulation. The extracts tested were distributed in Kahn tubes (11 x 75 mm) in serial dilutions (0.5 ml/tube); equal amount (0.5 ml) of freshly prepared 1% rbc was then added to each tube. Final concentration of rbc was 0.5%. Most tests were carried out at +4°C temperature. Several tests were carried out at room temperature (21°C), and a few in incubator at 37°C. Except for tests carried out at incubator temperature, where readings were made after 4 to 6 hours, in all other tests at refrigerator and room temperatures cells were allowed to settle overnight, and the results read next morning by the pattern method.

Results. Agglutinating action of leukemic passage A filtrates was consistent in dilutions up to 1:160 and sometimes up to 1:320 or 1:640, provided that heated (55°C ½ hr) extracts were used for the tests (Table I). Fresh leukemic filtrates had only occasionally a "questionable" agglutinating effect in either highest (1:20) concentration or, curiously, in one of the higher dilutions (1:80 or 1:160). Most fresh leukemic filtrates tested, however, were in all dilutions completely inactive on agglutination tests.

Heating of leukemic filtrates for ½ hour to 40°, 45°, or 50° was insufficient to bring out their full agglutinating potency. Heating to 55°C for 30 minutes was sufficient, and was used routinely. Extracts retained their agglutinating activity after heating at 60°C for ½ hour. In one experiment the extract was boiled 5 minutes, and still retained its agglutinating potency. Heating of leukemic filtrates to 55°C for ½ hour caused formation of a precipitate which could be readily sedimented by centrifugation at 3,000 rpm for 5 to 10 minutes. The resulting clear supernate was inactive on agglutination tests, whereas the resuspended sediment proved highly active.

C3H mouse erythrocytes were found most suitable, slightly more sensitive than those from Ak mice, and were used routinely for the tests. Guinea pig and rabbit erythrocytes were also agglutinated by heated (55°C ½ hr) leukemic extracts, but to a lesser extent.

Effect of temperature on agglutination. Agglutination tests were routinely carried out at +4°C. The heated (55°C ½ hr) leukemic filtrates agglutinated rbc also at room temperature (21°C); there was no elution of agglutinin. The tests appeared more sensitive, however, when carried out at +4°C. When a few tests were carried out at 37°C, agglutination also occurred, although in some tubes at least, it was accompanied by a slight hemolysis. Agglutinating potency of leukemic filtrate was not inactivated by leaving the extracts, prior to mixing with rbc, for several hours at room temperature. In one experiment the filtrate was kept at 21°C for 5 hours, then heated (55°C ½ hr) and finally mixed

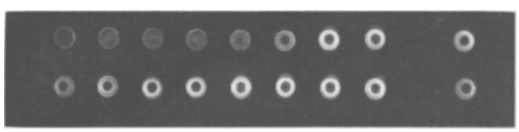

FIG. 1. Agglutinating action of heated (55°C, ½ hr) passage A leukemic filtrate on C3H mouse erythrocytes. Results read by pattern method, looking at bottom of tubes in mirror. Cells were allowed to settle overnight at +4°C temperature. Back row (top): heated extracts agglutinating in dilutions up to 1:320. Front row (bottom): fresh filtrates; no agglutination.

with rbc. In another experiment, the filtrate was first heated (55°C ½ hr), then left at 21° C for 5 hr and finally mixed with rbc. In both experiments agglutination resulted exactly as in simultaneous controls carried out with filtrates heated and mixed with rbc immediately after preparation.

Ultracentrifugation of leukemic filtrates prior to heating did not sediment the agglutinin. In 2 experiments, passage A leukemic filtrates were centrifuged in Spinco Model L ultracentrifuge at 0°C, using Swinging Bucket rotor SW 39, at top speed 40,000 rpm (average 125,000 x g) for 45 minutes. The resulting supernate was then heated (55°C ½ hr) and mixed with rbc. In both experiments ultracentrifugation did not affect agglutinating potency of leukemic extracts. Heated supernate was active on agglutination tests as the original extract. Since leukemogenic and oncogenic activity of extracts had been previously found to be sedimented after centrifugation for only 30 minutes at 40,000 rpm using rotor SW 39(4), it appears that agglutinin is separable from leukemic virus and related oncogenic agents present in the leukemic filtrates.

Filtrates from x-ray-induced-leukemia were tested, using either C3H donors with primary x-ray-induced-leukemia (150 r, total body, 4 times, at weekly intervals), or those with passage X virus induced leukemia(5). Two filtrates were prepared from primary x-ray-induced-leukemia, and both agglutinated when tested after heating (55°C ½ hr), none when fresh. Two passage X filtrates were tested, and both agglutinated, but only after heating, in dilutions to 1:160.

Filtrates from spontaneous Ak mouse leukemia were also tested. Fresh Ak filtrates had no agglutinating effect on either Ak or C3H rbc. Heated (55°C ½ hr) filtrates agglutinated in some instances only, and in higher concentrations. Of 10 filtrates tested, 3 did not agglutinate at all (Table I).

Filtrates from spontaneous C3H mouse mammary carcinomas were tested in 3 experiments, using for extracts either single mammary carcinomas, or several pooled mammary tumors. Neither fresh, nor heated (55°C ½ hr) filtrates had an agglutinating effect on C3H mouse erythrocytes.

Fresh filtrates from normal organs (liver, spleen, heart, lungs, kidneys, testicles) from young, healthy C3H(f) mice had no agglutinating effect. Of 9 heated (55°C ½ hr) normal organ filtrates tested, 5 agglutinated in dilutions 1:20, and 2 of these also in dilution 1:40 (Table I); the remaining extracts were inactive in all dilutions tested.

A filtrate was also prepared from normal C3H embryos; no agglutination resulted when either fresh or heated (55°C ½ hr) embryo filtrate were mixed with C3H erythrocytes.

Serum neutralization. In 3 experiments, serum obtained from a rabbit that had received several weekly injections of passage A leukemic filtrates was inactivated (56°C ½ hr). Normal inactivated rabbit serum was used in a simultaneous control experiment. Heated (55°C ½ hr) passage A leukemic filtrate was first tested for agglutinating potency and the highest dilution (usually 1:80 or 1:160) which still caused agglutination was determined; the last 2 highest dilutions of leukemic filtrate which were able to cause agglutination were then used for serum neutralization tests. Serial dilutions of either immune serum (0.25 ml), or normal serum in the simultaneous control series, were then mixed with equal quantities (0.25 ml) of proper dilutions of leukemic filtrate and incubated at room temperature for 1 hour. Mouse rbc (1%) were then added (0.5 ml), and test tubes placed in refrigerator. The results were read next morning by the usual pattern method. The immune rabbit serum in dilutions up to 1:128 neutralized the agglutinating potency of leukemic extracts whereas normal rabbit serum had a neutralizing effect only in 1:4, or at the utmost 1:16, dilutions.

Discussion. It is evident from these experiments that passage A leukemic filtrates, known to induce leukemia consistently, when inoculated into suckling mice of a susceptible strain, agglutinate C3H or Ak red blood cells *in vitro*. Curiously, this agglutinating potential of the leukemic filtrates becomes evident only after extracts have been heated (55°C ½ hr), *i.e.*, after their leukemogenic potency has been essentially destroyed. It is possible

to speculate that fresh extracts contain an inhibitor which can be inactivated by heating, not unlike mouse pneumonitis virus, which was also found by Mills and Dochez(6) to agglutinate murine erythrocytes, provided that lung extracts containing the virus, had been heated to 80°C for 5 minutes prior to agglutination test. Whether the agglutinating potency of leukemic extracts is actually related to presence of the leukemic agent, however, remains to be determined. If related, the agglutinin would be separable from the agent by ultracentrifugation. The agglutinin is relatively stable, and it does not elute. For that reason, agglutination tests could be carried out either at refrigerator, or at room temperatures, although the former appeared more suitable.

Filtrates from spontaneous Ak leukemia agglutinated only in some instances, and in higher concentrations. Since it was previously observed that on inoculation tests only some of the Ak filtrates were leukemogenic(4), the fundamental question remains as to whether the agglutinating potency of some of the Ak filtrates tested could be related to their ability to induce leukemia. In 3 experiments thus far performed, inactivated (56°C ½ hr) serum from a rabbit which had received several injections of passage A filtrates in view of immunization, inhibited the agglutinating potency of passage A leukemic filtrates. Such a serum had also a partially neutralizing effect on leukemogenic potency of the extracts(5).

In control experiments, fresh or heated (55°C ½ hr) filtrates from pooled normal organs, or mouse embryos, agglutinated only in some instances and in higher concentrations. A few filtrates prepared from spontaneous mammary carcinomas did not agglutinate.

In 1947 the author observed(7,8) that filtered extracts prepared from spontaneous mouse mammary carcinomas, a transplantable mouse sarcoma, and spontaneous or transplanted mouse leukemia, exerted a destructive action on mouse red blood cells *in vitro*. Many of the extracts tested, particularly those prepared from spontaneous mouse mammary carcinomas and mouse leukemias, hemolyzed mouse red blood cells after incubation at 37°C for 2½ hours. A clumping of red blood cells was also observed, particularly when extracts from tumors were used. All tests were made in incubator at 37°C, or at room temperature. Similar results were observed with extracts prepared from human tumors(9). All these tests were made with fresh tumor filtrates.

Agglutinating or hemolytic action of mouse tumor and mouse leukemia extracts was not consistent, however. Their agglutinating and/or hemolytic potency disappeared after extracts had been kept at room temperature for only a few hours. Furthermore, agglutinating and/or hemolytic potency of these extracts was evident only in high concentrations, in most instances not exceeding 1:10 or 1:20, and only when fresh extracts were tested. Normal organ extracts only occasionally showed a similar effect(7,8).

Salaman also reported(10) that mouse tumor extracts agglutinate red blood cells; rabbit erythrocytes were most sensitive, those of mice 4 to 16 times less sensitive. Again, this potency was very labile, disappearing in extracts left for only a few hours at room temperature. Furthermore, extracts prepared from normal organs showed a similar potency. These tests again were made with fresh extracts; their potency diminished rapidly after heating.

The agglutinating action of leukemic filtrates described here differs fundamentally from that observed in our previous experiments, or those carried out by Salaman. The main difference is that the agglutinin now described requires heating (55°C ½ hr) prior to mixing with rbc, and that it is relatively stable. It is unaffected when standing for at least 5 hours at room temperature, whereas those described previously were extremely labile. In Salaman's experiments, rabbit erythrocytes were most sensitive; in our studies, murine erythrocytes were considerably more sensitive to the agglutinating action of leukemic filtrates than those from either guinea pigs or rabbits.

It was recently reported by Eddy, Rowe and their associates(11) that the "polyoma" virus agglutinated guinea pig, hamster and human erythrocytes. The term "polyoma" was given by Stewart and Eddy to the parotid tumor virus originally isolated from

Ak leukemia, and identified in our laboratory in 1953(12), and later grown in tissue culture by Eddy and her associates(13). The main differences between agglutinating property of the parotid tumor virus,† and that of leukemic filtrates, are as follows: a) the parotid tumor virus agglutinates‡ when either fresh or heated (60°C ½ hr), whereas leukemic filtrates agglutinate only after heating (55°C ½ hr); b) the parotid tumor virus elutes, and for that reason the tests must be carried out at refrigerator temperature, whereas the leukemic agglutinin described here does not elute; c) murine erythrocytes are more sensitive than those of guinea pigs to the agglutinating action of leukemic filtrates.

Summary. 1) Heated (55°C ½ hr) passage A leukemic filtrates had an agglutinating effect on mouse erythrocytes in dilutions up to 1:160, occasionally higher. Fresh filtrates did not agglutinate. 2) Agglutinin could be separated from leukemic agent by ultracentrifugation. 3) Tests were done at +4°C but could also be carried out at room temperature; the agglutinin did not elute. 4) Heated (55°C ½ hr) filtrates from spontaneous Ak leukemias agglutinated only in some instances, and in higher concentrations. Whether the agglutinating potency of some Ak leukemic filtrates is related to their ability to induce leukemia, remains to be determined. 5) Fresh or heated filtrates from pooled normal mouse organs agglutinated only in some instances, and in higher concentrations. 6) The agglutinating potency of heated leukemic passage A filtrates could be inhibited by specific rabbit immune serum to 1:128 dilution. Normal rabbit serum had an inhibiting effect only in 1:4 to 1:16 dilutions.

† This refers to *tissue-culture-grown* parotid tumor virus, which agglutinates rbc *in vitro*, and also induces high incidence of parotid, and other tumors, following inoculation into newborn mice. Curiously, however, fresh filtrates prepared *from parotid tumors,* do not agglutinate, and only occasionally induce parotid, and other tumors on inoculation tests.

‡ After this manuscript was completed, the activation of hemagglutinin in certain low-potency preparations of parotid tumor (polyoma) virus, by heating to 56°C ½ hr, was also reported (Hartley, J. W., and Rowe, W. P., *Virology,* 1959, v7, 249).

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Hyperlipemia and Hemolysis I. Interaction of Sodium Oleate with Human Erythrocytes. (24851)

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The lytic action of fatty acids on washed mammalian erythrocytes suspended in isotonic saline has long been recognized, although the precise mechanism remains speculative(1,2). Lysis of the same erythrocytes, when suspended in homologous plasma, requires significantly higher concentrations of fatty acid since normal plasma contains potent inhibitors of fatty acid hemolytic activity(3-6). Despite this marked hemolysis-inhibitory property of plasma *in vitro,* evidence has accumulated that *in vivo* hemolysis