

IMMUNOLOGICAL RELATIONSHIPS OF A FILTERABLE AGENT
CAUSING A LEUKEMIA IN ADULT MICE

I. THE NEUTRALIZATION OF INFECTIVITY BY SPECIFIC ANTISERUM*

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A neoplastic disease of the hematopoietic system of mice which is transmissible by cell-free filtrates has been described in a previous report (1). This lethal disorder, first noted in a Swiss mouse injected ineffectually with a cell-free preparation of the Ehrlich carcinoma, has the character of a leukemia and is serially and regularly transmissible to both Swiss and DBA/2 adult mice by an agent present in cell-free filtrates prepared from the neoplastic tissue.

Antisera capable of neutralizing the agent have now been produced in mice and rabbits, and the effect on the agent of various normal sera and those from mice with other neoplasms, including leukemia, have also been subjected to inquiry. An active immunity to the leukemic effects of the virus has been regularly induced in mice by a vaccine prepared by treating filtered extracts of the neoplastic tissues with formalin. These results are here reported.

Materials and Methods

Animals.—Adult mice of Swiss breed weighing 16 to 19 gm. were used throughout.

Antigen.—The greatly enlarged spleens of Swiss mice with leukemia as a result of inoculation with the agent 21 to 35 days before were ground in a TenBroeck homogenizer. A 20 per cent suspension was made with Locke-Ringer's solution,¹ and this was centrifuged for 10 minutes at 1,000 R.P.M. to remove coarse particles. The supernatant fluid contained whole cells in substantial numbers. In some instances, it was employed as the antigen without further treatment, and in others, after passage through a Selas 03 filter impermeable to *Escherichia coli*.

Antisera.—Four rabbits weighing 4 to 5 kilos were immunized. Two were inoculated with filtrates and two with cell suspensions of leukemic spleens. A course of four intraperitoneal injections of 5 cc. each was given at 3-day intervals. The rabbits were bled 14 to 21 days after the last injection, and the sera thus obtained were tested individually.

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¹ Locke-Ringer's solution, buffered at pH 7.2, contained 6 mg. of glucose per ml., 50 μ of penicillin per ml., and 2 mg. of streptomycin per ml.

In addition, 50 Swiss mice, weighing 25 to 28 gm., were bled. Their sera were collected between 30 and 60 days after two or three intraperitoneal injections of 0.2 cc. each of filtrate given at 2-week intervals. These sera were pooled.

All sera were stored in sealed ampoules at 4°C. with no preservative added.

Neutralization Tests.—All sera were inactivated in a water bath at 56°C. for 30 minutes. Undiluted serum was mixed in equal amount with either a freshly prepared 20 per cent suspension of the tissue of leukemic spleens or the filtrate from such material. After incubation in a water bath at 37°C. for 90 minutes, 0.2 cc. of the mixture was injected i.p. into each test mouse.

Vaccination.—Enough formalin was added to filtrates of 10 or 20 per cent extracts of infected spleens to give a final concentration of 1:500. The mixture was kept at 4°C. for 21 days and shaken manually each day throughout this period prior to use. It was then

TABLE I
Resistance of Mice to Reinoculation with the Agent

Original inoculum*	Challenged with†	Time of challenge (mos. after 1st inoculation)				Total	
		4	6	8	10	No. positive No. inoculated	Per cent positive
Cells	Cells	2/14	1/12	0/6	—	3/32	9.4
	“	5/5	4/5	5/5	—	14/15	93.3
Cells	Filtrate	1/5	0/4	—	—	1/9	11.0
	“	5/5	5/5	—	—	10/10	100
Filtrate	Cells	2/23	0/3	4/12	6/25	12/63	19
	“	5/5	5/5	5/5	5/5	20/20	100
Filtrate	Filtrate	—	0/3	0/10	0/3	0/16	0
	“	—	5/5	5/5	5/5	15/15	100

* 0.2 ml. 10 per cent dilution, intraperitoneal.

† 0.2 “ 20 per cent “ , “

tested as a vaccine. Three i.p. injections were given at weekly intervals to mice weighing 16 to 19 gm. prior to challenge with active material at times indicated in the text.

RESULTS

When cell-free material was first proven to be effective in inducing the disease, approximately 20 per cent of the mice injected with whole cells or filtrate remained healthy (1). These survivors, which had never shown any signs of leukemia, were subsequently found immune to a challenge injection of leukemic material that caused the disease in most of the untreated control mice. This proved to be the case even when months had elapsed between the original and the challenge inoculations (Table I).

As can be seen, most of the mice which had survived the original injection failed to develop leukemia when reinjected with another disease-producing, cell-

free filtrate or cell suspension. Of particular interest was the fact that animals which had survived injection with filtrate also proved highly resistant on subsequent inoculation of either filtrate or neoplastic cells.

Although the results of these experiments indicate that the mice surviving reinoculation had a manifest resistance as compared with the controls, the source of their immunity was not clear. It was necessary to find out whether they were innately unsusceptible and hence had survived the first as well as the second inoculation, or whether the small amount of the agent originally injected had been sufficient to cause an immune response but not to induce the disease. With this aim, the antigenicity of the agent was investigated.

Neutralization Tests.—Neutralization tests were conducted with the antisera from mice and rabbits injected with the agent, as described under Methods. Sera from the rabbits obtained prior to immunization and sera from normal mice served for controls.

TABLE II
Neutralization of Agent by Specific Mouse Antiserum*

	Normal serum		Antiserum	
	$\frac{\text{No. positive}}{\text{No. inoculated}}$	Per cent survivors	$\frac{\text{No. positive}}{\text{No. inoculated}}$	Per cent survivors
Filtrate.....	20/24	16.7	3/30	90
Cells.....	24/25	4.0	10/25	60

* Serum mixture incubated at 37°C. for 1½ hours before inoculation of 0.2 cc. intraperitoneally.

The results of testing the pooled serum collected from mice immunized with filtrate are given in Table II. Although sera from normal mice, whether from strains susceptible or resistant to the agent, do not contain neutralizing antibody, antisera from the treated mice neutralized the agent.

When the antiserum was mixed with filtrate, 90 per cent of the thirty mice survived, as compared with 16 per cent of those that had received normal serum plus filtrate. When cells were tested with antiserum, 60 per cent of the mice survived as compared to 4 per cent among the normal serum plus cells controls. Furthermore, the incubation period of the disease in the mice which did develop leukemia from the immune serum-cell mixture was on the average 21 to 28 days, as compared to 10 to 14 days in the mice given the cells with normal serum.

Antisera prepared in rabbits gave similar results. The findings with the serum from rabbits 1 and 2, which were immunized with cell-free filtrates, appear in Table III. When the pre-immunization serum of rabbit 1 was mixed with the leukemic spleen cell suspension, 15 per cent of the 45 control mice

survived the inoculation, as compared to 72 per cent of the 44 mice given the cells in immune serum. When the normal serum was mixed with filtrate, 14 per cent of the 50 mice survived, whereas all of the 51 mice injected with the mixture of antiserum and filtrate lived.

None of the mice survived the inoculation of the mixture of cells and the control serum of rabbit 2, whereas there was a survival rate of 67 per cent among the 18 mice given the cells in the immune serum from the same rabbit. When the filtrate was tested, 12 per cent of the 86 control mice lived, as compared to 95 per cent among the 158 mice given the antiserum-filtrate mixture.

Essentially the same results were obtained with the serum from rabbits immunized with cell-containing infected spleen preparations. This is shown in

TABLE III

The Effect of Antiserum from Rabbits Inoculated with Filtrate on the Infectivity of Leukemic Cells or Cell-Free Filtrate

	Rabbit 1	Rabbit 2	Total
Cells mixed with normal serum			
No. positive/No. inoculated	38/45	18/18	56/63
Per cent survival	15.6	0	11
Cells mixed with antiserum			
No. positive/No. inoculated	12/44	6/18	18/62
Per cent survival	72	67	70
Filtrate mixed with normal serum			
No. positive/No. inoculated	43/50	75/86	118/136
Per cent survival	14	12	13
Filtrate mixed with antiserum			
No. positive/No. inoculated	0/51	8/158	8/209
Per cent survival	100	95	96

Table IV. When the normal pre-immunization serum of rabbit 3 was mixed with cells, 15 per cent of the 20 mice survived, as compared to 60 per cent among those inoculated with the immune serum mixture. When the filtrate was used, 21 per cent of the controls survived, as compared to 100 per cent in the antiserum group.

When cells were mixed with normal serum from the 4th rabbit, 29 per cent of the 59 inoculated mice survived, as compared with 86 per cent among those receiving the antiserum mixture. With filtrate, 15 per cent of the controls survived, in contrast to 100 per cent of those given filtrate plus antiserum.

From these data, there appears to be no significant difference in the neutralizing capacity between the antisera obtained from rabbits after immunization with either cells or filtrate. Whatever differences have been observed may be attributed either to the variation of the individual rabbit to produce antibody,

or to the variation in the virulence of the leukemic preparation used in each test, as can be seen by noting the controls in each of the tables. In all instances, however, the filtrate was more effectively neutralized than the cell suspensions.

Further evidence indicating that neutralization of infectivity is brought

TABLE IV

The Effect of Antiserum from Rabbits Inoculated with Cell Suspensions on the Infectivity of Leukemic Cells or Cell-Free Filtrate

	Rabbit 3	Rabbit 4	Total
Cells mixed with normal serum			
No. positive/No. inoculated	17/20	42/59	59/79
Per cent survival	15	29	25
Cells mixed with antiserum			
No. positive/No. inoculated	8/20	8/59	16/79
Per cent survival	60	86	80
Filtrate mixed with normal serum			
No. positive/No. inoculated	15/19	17/20	32/39
Per cent survival	21	15	18
Filtrate mixed with antiserum			
No. positive/No. inoculated	0/28	0/20	0/48
Per cent survival	100	100	100

TABLE V

The Effect of Untreated and Absorbed Antiserum on the Infectivity of Leukemic Cells or Cell-Free Filtrate

Rabbit serum	Incubated with cells*		Incubated with filtrate*	
	$\frac{\text{No. positive}}{\text{No. inoculated}}$	Per cent survival	$\frac{\text{No. positive}}{\text{No. inoculated}}$	Per cent survival
Normal control	9/9	0	8/9	13
Leukemic				
Anti-filtrate	4/10	60	0/9	100
Absorbed anti-filtrate	4/9	56	0/8	100
Anti-cells	3/9	67	0/9	100
Absorbed anti-cells	3/9	67	0/9	100

* Serum mixtures incubated at 37°C. for 1½ hours before inoculation of 0.2 cc. intraperitoneally.

about by specific antiviral antibody was obtained from studies with absorbed antiserum. In one experiment, shown in Table V, each sample of antiserum was divided into two aliquots. One was set aside and the other was extensively absorbed with dried normal mouse spleen and serum before testing. As can be seen, the absorption procedure did not remove the neutralizing antibody. Such treated antiserum was as effective as the unabsorbed. None of the mice inocu-

lated with the cells in normal rabbit serum survived, whereas between 56 and 67 per cent of the mice injected with the cells in absorbed or untreated anti-serum lived. Similarly, only 13 per cent of the mice given the filtrate-normal serum survived, as compared to 100 per cent of those injected with the filtrate-antiserum mixtures. The antisera prepared against filtrate and against cells are listed separately to demonstrate that each retains the capacity to neutralize infectivity after absorption with normal tissue. It remains to be determined whether absorption with leukemic cells will remove neutralizing antibody. This experiment is in progress.

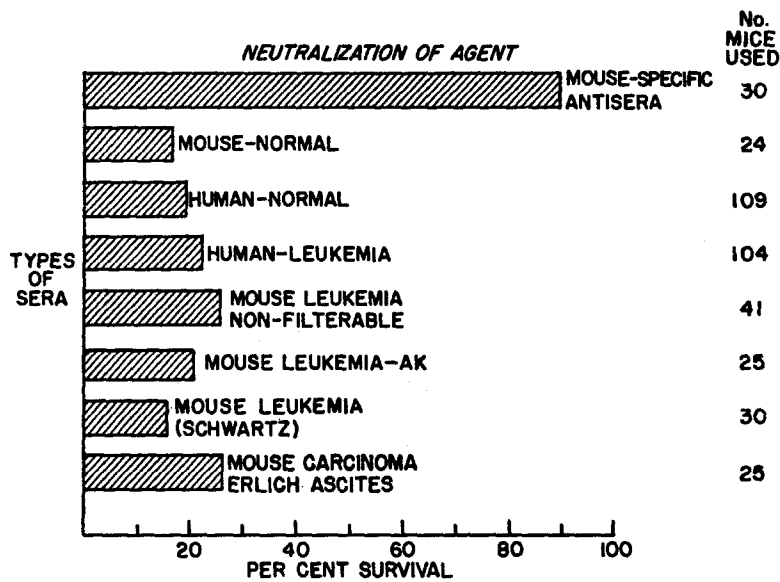


FIG. 1

Neutralization Tests with Other Sera.—A number of samples of human serum were tested for neutralizing power (Fig. 1). Of the normal sera, eight were from individual donors to the Blood Bank of Memorial Hospital and three others were pooled sera, kindly sent to us by Dr. Thomas Francis, Jr. A total of 20 per cent of the 109 mice inoculated with the mixtures of these sera and our leukemia agent survived. Eleven sera from as many leukemic human beings² were also studied. Two of the samples were from patients in remission and the others from patients with either acute or chronic leukemia. There was an over-all survival rate of 23 per cent of the 104 mice used in these tests, as com-

² The sera from leukemic human beings were received through Drs. R. R. Ellison, L. Murphy, and C. Tan of the Leukemia Service of Memorial Hospital.

pared to 22 per cent among the controls. In other words, the percentage of survivors was about the same as if serum had been absent.

L1210, C1498, 82T, and 8174 are non-filterable, well established cell-transmissible mouse leukemias. Sera from mice in the early as well as advanced stages of one or another of these cell-transferred leukemias contained no neutralizing antibody. The total survival rate of the 41 mice inoculated with these serum-agent mixtures was 25 per cent, as compared to 20 per cent for the controls.

The lack of effect of the sera of leukemic AK mice is especially noteworthy. Gross and others have reported leukemia induction in newborn mice injected with cell-free material from tissues of leukemic mice of this strain (2-4). Hence, it was of interest to determine whether an immunologic relationship existed between the AK leukemia agent and that of our disease. Only 20 per cent of the 25 mice inoculated with the sera of leukemic AK mice plus the agent survived, indicating that these sera contain no protective antibody against our agent. Further evidence to support this finding was provided by data not given here which show that rabbit antiserum against the rapidly passaged AK agent (5) supplied by Dr. Ludwik Gross, and rabbit antiserum against the Stewart and Eddy tissue-cultured agent (6) supplied by Dr. Sarah Stewart, are also lacking in neutralizing antibody.

Swiss mice bearing yet another leukemia induced by cell-free material (7) were obtained from the laboratory of Dr. S. O. Schwartz. The sera of these animals did not neutralize the infectivity of our agent, only 5 of the 30 mice surviving.

Since our leukemia agent was originally recovered from a mouse which had been inoculated in infancy with a cell-free extract of the Ehrlich ascites carcinoma (1), the sera from mice bearing this tumor were also tested for neutralizing capacity. None was demonstrable, only 6 of the 25 mice surviving.

The results with all the sera tested are shown in Fig. 1, in terms of the per cent survival of mice inoculated with each of the various serum-filtrate mixtures. These are compared with the survival rate of 16 per cent among the 24 control mice which had received normal mouse serum plus the agent and of 90 per cent among the 30 mice which were injected with the specific antiserum-agent mixture. From the data it would appear that there is no direct immunological relationship between any of the sera tested and our leukemia agent of Swiss mice.

Vaccination against the Agent.—Since the filtrate of leukemic tissue was capable of inducing a good neutralizing antibody response in rabbits and mice, the possibility of preparing a vaccine from the agent which would induce a specific active immunity was investigated. One hundred mice were immunized with a formalin-treated filtrate. Each received three intraperitoneal injections of 0.3 cc. each, given at weekly intervals, as describe under Methods. On the

7th, 14th, 21st, and 28th days after the last injection, 25 of the treated mice were challenged by an intraperitoneal injection of 0.2 cc. of a 20 per cent filtrate of leukemic spleens. An equal number of normal animals of the same age and sex had been set aside at the beginning of each experiment to serve as controls for the challenge inoculation given the vaccinated group.

The results of these four experiments are presented in Fig. 2. Among the mice challenged 7 days after the last vaccine injection 80 per cent survived, as contrasted with 14 per cent among an equal number of the control mice. At 14

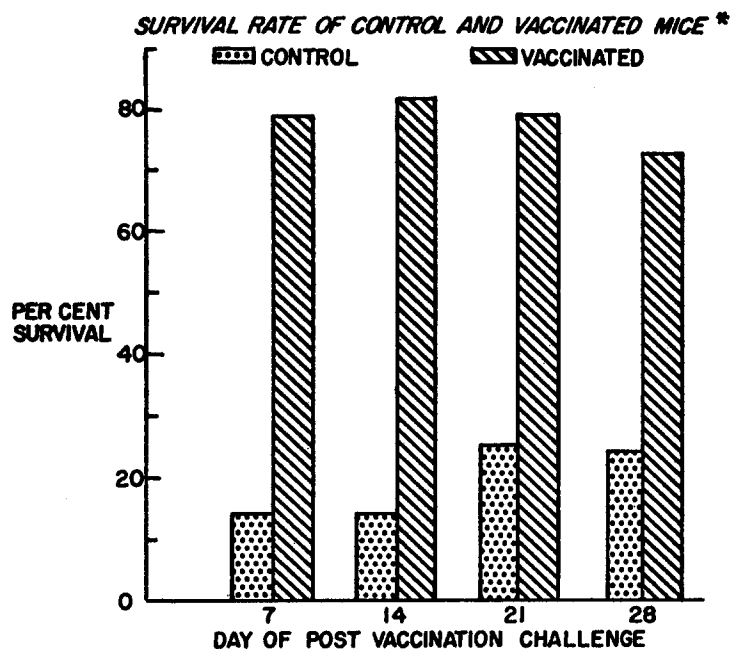


FIG. 2

days after, 82 per cent of the immunized animals lived, as compared with 14 per cent of the controls. After 21 days, 79 per cent of the mice in the vaccinated group and 25 per cent of the controls survived; and of those tested after 28 days, 78 per cent survived, as compared with 23 per cent of the controls.

Vaccination with formalized filtrates also prevented leukemia in mice inoculated with whole leukemic cells. Of 30 immunized mice, only 7 came down with the disease after intraperitoneal challenge with a 20 per cent cell suspension from leukemic spleens, whereas 79 per cent (19 mice) of the 24 unvaccinated controls developed it.

Effect of Immunization on Other Mouse Neoplasms.—Since a strong immunity

against the leukemia was obtained by means of the vaccine, it was decided to test its protective capacity against a number of other mouse neoplasms.

Leukemias C1498 in C₅₇B1/6 mice, 82T in F₁(C₅₈ × BALB) mice, and L1210 in DBA/2 mice, and also the Ehrlich ascites carcinoma in Swiss mice, were selected for test. Adult C₅₇B1/6 and the F₁ mice were injected with freshly prepared 20 per cent suspensions of leukemic spleen, since these mice are not susceptible to the agent (1). The DBA/2 and Swiss mice were vaccinated with formalin-inactivated filtrates. Three intraperitoneal inoculations of 0.3 cc. each were given per group. Fourteen days after the last of the injections, the mice received the test material.

Although specific neutralizing antibody against our agent could be demonstrated in the sera of the immunized animals, no protection was evident against the other lines of transplantable leukemia, nor were they altered in their course

TABLE VI
The Effect of Vaccination against Other Mouse Neoplasms

Neoplasm	Mouse strain	Control	Immunized
		$\frac{\text{No. positive}}{\text{No. inoculated}}$	$\frac{\text{No. positive}}{\text{No. inoculated}}$
C1498	C ₅₇ B1/6	20/20	20/20*
82 T	F ₁ (C ₅₈ × BALB)	10/10	10/10*
L 1210	DBA/2	8/8	8/8‡
Ehrlich (SC)	Swiss	7/10	8/10‡

* Vaccinated with viable material.

‡ Vaccinated with formalinized material.

(Table VI). Both control and immunized mice died of the test leukemias. In the groups with the Ehrlich ascites tumor the number of cells implanted (200,000 cells, one-fifth of the usual dose) was so small that survivors had been expected. Among the controls 70 per cent developed tumors, as compared with 80 per cent of the immunized mice. When vaccinated mice were injected with 500,000 cells intraperitoneally, there were no survivors either among them or the controls.

Effect of Immunization with Other Cell-Free Leukemic Agents.—Although it was known that the sera from AK leukemic mice did not neutralize the infectivity of our agent, it seemed of interest to determine whether active immunization with the AK agent would have some effect.

Two groups of 20 Swiss mice each were injected—one with cells, and the other with filtrates of a potent AK leukemia suspension.³ Two intraperitoneal

³ The AK preparation kindly provided by Dr. George Woolley of the Sloan-Kettering Institute was active in inducing leukemia and parotid gland tumors when inoculated into infant C₂H mice.

injections of 0.2 cc. each were given at 3-day intervals to each group. A third group of ten mice was set aside for controls. Ten days after the last injection the mice of all three groups were challenged intraperitoneally with 0.2 cc. of a 20 per cent filtrate containing our agent. There was no difference in the results with the control and "immunized" groups. Eighty per cent in each case developed the disease.

A similar experiment was set up with 16 mice that had survived inoculation with the leukemia of Schoolman *et al.* (7). Eighty-three per cent of these mice died of leukemia after being challenged with our agent, a figure essentially similar to that for our control animals.

DISCUSSION

There have been recent reports on the immunological aspects of two other leukemia agents. Dulaney *et al.* (8) found that sera prepared by treating either mice or rabbits with the AK leukemia agent did not contain neutralizing antibody for the agent. When the mixtures of anti-serum and AK extract were inoculated into AKR or C₃H/Hf mice, there was no significant difference in the onset of leukemia between the control and experimental groups. In addition, old mice which had received one or more injections of the AK agent when newborn and had remained apparently healthy came down with leukemia after receiving small amounts of AK leukemic cells.

In contrast, Graffi *et al.* (9) reported a significantly lower percentage of leukemia developing in newborn mice inoculated with the leukemia agent they obtained from Sarcoma I (Sa I), mixed with rabbit antiserum against cell-free extracts of the Sa I, than in the controls which had received the agent, either alone or mixed with normal rabbit serum. It was not possible, however, to detect neutralizing antibody in the sera of mice after immunization with Sa I filtrates "attenuated" by heat or formaldehyde. Thus, neither the AK nor Sa I leukemia agents appear to be antigenic in their natural host, the mouse.

In contrast to the findings obtained with the AK and Sa I agents, the leukemia agent of Swiss mice has been shown to evoke so good a specific antiviral response in both mice and rabbits as to make the production of a prophylactic vaccine feasible.

Specific antibody neutralized the infectivity of both cells and cell-free preparations of infected spleens, although neutralization of the cells was not as complete as that of the filtrate. A similar phenomenon was observed in the protection of immunized mice against challenge with cellular suspensions of leukemic spleen. In the light of the studies of Rous *et al.* (10) on the failure of immune sera to affect living cells of the Shope fibroma of rabbits, it is difficult to reconcile this apparent effect of antibody on virus-containing leukemic cells. Further studies are being made to determine whether the cells were viable, for it

seems possible that they are so fragile as to have been injured by the grinding prior to suspension, and therefore had allowed the entry of antibody.

Work is in progress to define the duration of the immunity resulting from vaccination and the effectiveness of a vaccine formalinized for a shorter period of time. The effect of the vaccine given at closer intervals, for instance at 3-day instead of the present 1-week intervals, is also being investigated.

SUMMARY

The antigenic character of a filterable agent which induces leukemia in adult mice has been investigated. Mice and rabbits injected with filtrate yielded sera which specifically neutralized infectivity of the agent. Normal sera, sera from mice with other neoplasms—including leukemias from which no causative agent has as yet been obtained,—and sera from leukemic human beings contained no such neutralizing antibody. A formalinized vaccine prepared from filtrates of leukemic spleens induced a significant degree of immunity against the agent, approximately 80 per cent of the vaccinated mice being immune on challenge with it.

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