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Character of Agent Inducing Leukemia in Newborn Mice.* (22697)

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It is now well established that there are filterable agents capable of causing leukemia or tumors, or both, in several species of animals, and Stanley(1) concluded that many, if not all, tumors in man as well as in animals could be caused by viruses. Of current interest is the virus‡(2) in tissues of high leukemia strains of mice that will induce leukemia when injected into mice of low-leukemia strains less than 1 day old. Technical difficulties have greatly handicapped confirmation and research on the nature of this agent: the necessity of using animals less than 12 hours of age; the extraordinary host specificity; the long latency period (about 7-12 months) which is close to that of spontaneous leukemias; the variable and usually small percentage of leukemias induced; the necessity of using large numbers of controls to ascertain that the tumors were induced and not spontaneous; and the performance of bioassays to further exclude contamination of the inoculum with living cells.

Two sets of experiments are reported here. The first was initiated with Dr. Ludwik Gross. Motivated by the thought that if a virus causes leukemia it may play a determining role in leukemia induction by radiation, Dr. Gross was invited to Oak Ridge in July, 1953, to demonstrate his technic. The second set of experiments were set up in Boston primarily to establish the sensitivity of different low-leukemia strains to the agent in Ak tissue. The immunogenetic character of many leukemias was tested by transplantation assays, since one or a few immature or leukemic cells from Ak donors may have been present in the inoculum and may have survived when grafted in newborn mice, giving rise to leukemia close to the "leukemia age." It was assumed that virus-induced leukemias have the immunogenetic character of the recipient strain, and cell-induced leukemias that of the donor strain.

Materials and methods. Strains of mice. Low-leukemia strains, C3H (abbr. H), LAF_1, DBA/1, BALB/c, Rf, and C57BR, were used. The tissue extracts containing the agent were prepared from Ak leukemic mice. Sublines of Ak and H mice are indicated as follows: a = Andervont, g = Gross, r = Russell, b = Bittner, R = Rockefeller Institute (Lynch), o = Oak Ridge, eb = mam-

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‡ The terms virus and agent are used interchangeably for the substance in tissue extracts capable of inducing or hastening the development of leukemia.

TABLE I. Results of Injections of Ak Leukemia Extracts into Newborn Mice.

Donor		Recipients					
Strain	Material	Ha	Hg	Hr	Hb/Hr	Total No.	%
Ak/g	Centrifugate	2/5*	—	3/25	—	5/ 30	16.7
"	Filtrate	4/16	3/4	1/34	2/11	10/ 65	15.4
Ak/o	"	1/2	—	1/17	5/19	7/ 38	18.4
AkR	Centrifugate	—	—	4/6	—	4/ 6	66.7
	Total No.	7/23	3/4	9/82	7/30	26/139	
	%	34.8	75.0	11.0	23.3		18.6
	Control No.	1/10	0/15	4/45	7/34	12/104	
	%	10	0	8.8	20.6		6.7

* No. positives (leukemias) over total No. of mice inj.

mary agent-free line of the Roscoe B. Jackson Memorial Laboratory. The use of the indicated sublines of C3H and Ak as both source of the agent and host was by necessity. *Preparation of extracts.* The technic of Gross was slightly modified in the second experiment. Extracts of leukemic tissue were prepared from a recently isolated strain of transplantable AkR leukemia. Spleen, lymph nodes (not including cervical nodes in the second experiment because of their proximity to the salivary gland which may be carrier of another virus), thymus, and a small piece of liver were ground in a mortar with enough cold normal saline to make a 20% suspension, centrifuged at 3000 rpm for 10-15 minutes in a refrigerated centrifuge set at 0°C, and the supernatant fluid drawn off. The supernatant fluid was recentrifuged at 0°C at 3000 rpm for 15-30 minutes or 7000 g for 10 minutes, and the supernatant fluid again drawn off. In the second experiment, the floating fat was removed with a sterile swab. The extract was kept on ice in the refrigerator and used within 24-30 hours after preparation. Filtration was omitted in the second set of experiments in order to avoid further weakening of the extracts and because emphasis was placed on the character of induced leukemic cells. Instruments and equipment were chilled. *Inoculations.* Babies less than 12 hours old, and some 12-24 hours old were injected subcutaneously in the back with 0.05-0.1 cc of inoculum through a 27-gauge needle. In some instances, supplementary intraperitoneal injections were made to make a total dose of 0.1 cc. Implants of leukemic cell suspension in adults were made intramuscularly in the thigh. *Termination of experiments.* In the first experiment, nearly all mice were observed until natural death. The second experiment was terminated when the last surviving animals were 10-15 months of age (about 38% of the total number being younger than one year). This seemed permissible because of our limited objectives, the spontaneous leukemias in question being predominantly thymic and appearing mostly under 10 months of age.

Results. First experiment. The results of the first set of injections of Ak leukemia extracts into newborn mice (all less than 12 hours old) are summarized in Table I. In one subline (Hb/Hr), the incidence of leukemia among the controls was almost as high as in the experimental group and higher than the average in the various H sublines used. In subline Hr the difference between experimental and control groups was also slight. Transplantation assays were made on available mice in ignorance of the marked subline differences among C3H (=H) mice (Table II). On first glance, the results appear to

TABLE II. Transplantation Assays of 4 Leukemias Developing in Agent Injected Mice.

Donor			Recipient*		
No.	Strain	Material	AkR	Ak/o	Hr
Hb/Hr 26	Ak/o	Filtrate		0/ 6	0/11
10	Ak/g	"		0/ 4	1/ 5
Hr 47	AkR	Centrifugate	0/20		12/12
45	"	"		0/ 4	1/ 6
		Total	0/20	0/14	14/34

* Each recipient received intraper. 0.1 ml of a leukemic cell suspension, and was observed until natural death or for a period of at least 3 mo after inoculation.

TABLE III. Induction of Leukemia in Newborn Mice with AkR Leukemic Tissue Extract.

Strain	Material†	No. survivors 4 mo*	Leukemia		Months postinjection							
			No.	%	4–5	5–6	6–7	7–8	8–9	9–10	10–11	11–15
LAF_1	E	53	0									
	S	66	0									
Heb	E	54	8	15	3	1		1	2		1	
	S	42	0									
DBA/1	E	47	9	19		1	1	1	1	1		4
	S	35	0									
BALB/c	E	33	1	3			1					
	S	33	0									
Rf	E	29	10	38	1	2	2	2	1		2	1
	S	43	0									
C57BR	E	55	1	2							1	
	S	53	0									
AkR	E	34	26	76	2	3	5	6	3	3	3	1
	S	21	10	48			1	1	1	4	2	1

* Discounting from total a few mice on which adequate autopsy could not be performed.
† E = Extract; S = Saline.

confirm the findings of Gross in that 3 of 4 leukemias could be grafted on H mice, none on Ak mice, and one on neither strain. However, only one (Hr47) had a distinctly H (Hr) type of leukemia, but in this subline the difference in incidence of leukemia between agent-injected and control mice was negligible; hence the disease might have been spontaneous. The two leukemias arising in the Hb/Hr subline, which one would expect to be transplantable to all Hr mice, took in only 1 of 16 animals. Thus these findings cannot be taken as evidence that these leukemias were of the recipient immunogenetic types and hence induced by a virus. Interpretation of these results calls for ascertaining the degree of homozygosity and the immunogenetic relationship of the various sublines.

Second experiment. Leukemia induction. It seemed desirable to search for a low-leukemia strain with high sensitivity to the Ak agent, preferably a strain with less immunogenetic relationship with Ak as is known to exist between some sublines of C3H and Ak (3,4). Accordingly, newborn mice of 7 strains were injected with tissue extracts from mice with AkR leukemias (Table III). The extracts were presumably cell-free, and transplantation assays (Table V) suggest that most induced leukemias were certainly not induced by contaminating cells.

Four strains of animals proved fairly sensitive to induction of leukemia with Ak agent: Heb, DBA/1, Rf, and the high-leukemia strain, AkR, from which the agent was obtained (Table III). Leukemia was induced in 15% of a breast tumor agent-free subline of C3H (Heb) mice; none occurred among the controls. (Sublines of greater sensitivity were not available.) Leukemia was induced in 19% of DBA/1 mice; none occurred among the controls.

Of the low-leukemia strains, Rf proved most susceptible; 38% of agent-injected mice developed leukemia, at a time when none of the controls had it. Originally, the Rf strain had only 2-3% spontaneous leukemias, and it had no genetic relation to the Ak strain (3). In recent years, a small percentage of Rf mice "take" Ak leukemic cells. The spon-

TABLE IV. Transplantation Range of AkR Leukemic Cells.

Recipient strain	Leukemia	
	Adults	<1 day old
LAF_1	0/ 11	4/ 5
Heb	0/ 27	
DBA/1	0/ 7	
BALB/c	0/ 11	
Rf	17/174	
C57BR	0/ 8	12/13
AkR	18/ 19	10/10

One spontaneous leukemia in an Rf mouse was grafted on Rf and AkR mice with results as follows: Rf hosts 6/6, AkR hosts 0/5.

TABLE V. Transplantation Assay of Leukemias of Mice Injected with AkR Extracts.

Leukemia donor		Original passage				Subpassage I				Subpassage II			
		In strain of origin		In AkR		In strain of origin		In AkR		In strain of origin		In AkR	
No.	Age†	Result	Latency‡	Result	Latency	Result	Latency	Result	Latency	Result	Latency	Result	Latency
Heb 17	141	*2/6	55, 93	1/6	97	7/11	29–37						
						3/ 6	36	*1/6	83	0/5		5/5	44–48
20	141	*4/4	21–45	1/6	84	17/17	16–21	*6/6	23–28	4/4	15–37	4/4	14–15
						19/19§	19–20						
16	251	0/5		2/5	60, 205								
Rf 55	163	0/3		4/5	92–183								
56	163	3/5	16–18	1/5	69								
57	262	5/5	18–60	2/6	59, 108								
7	217	*4/6	27–42			1/ 4	44	0/4					
				*3/6	83–100			3/3	29–33				
12	143	2/6	136, 159	1/5	137								
16	237	0/6		0/5									
DBA/1 1	435	2/5	53, 82	0/3									
8	428	1/5	74	0/4									
9	428	*5/5	45–49	0/4		5/ 5	18–20	0/5					
BALB/c 13	186	*9/9	17–19	0/8		1/ 3	48	0/4					

* Indicates group from which subpassage was made. All AkR recipients were 1½-2½ mo of age when grafted.
† In days, when mouse was sacrificed for transplantation.
‡ No. of days after graft.
§ Frozen cells from same donor as transfer above.

TABLE VI. Mortality Data on Newborn Mice Injected with Leukemia Extracts (Second Experiment).

Strain	Material	No. inj.	No. weaned	% loss	No. survivors 4 mo	Mortality					Total mortality, 4-10 mo					
						1–4 mo		4–10 mo			Leukemia		Other causes		Combined	
						No.	%	4–6	6–8	8–10	No.	%	No.	%	No.	%
LAF_1	E*	87	59	32	54	5	8†	1	0	0	0		1	2‡	1	2‡
	S*	110	66	40	66	0		0	0	0	0		0		0	
Heb	E	112	62	45	55	7	11	9	9	6	7	13‡	17	31	24	44
	S	100	51	49	43	8	16	2	2	0	0		4	9	4	9
DBA/1	E	155	55	65	50	5	9	4	7	6	5	10	12	24	17	34
	S	138	38	71	38	0		5	0	3	0		8	21	8	21
BALB/c	E	54	39	28	34	6	13	2	6	1	1	3	8	23	9	26
	S	52	39	25	33	6	15	1	3	2	0		6	18	6	18
Rf	E	148	42	72	29	13	31	4	6	2	8	27	4	14	12	41
	S	112	49	56	43	6	12	0	0	0	0		0		0	
C57BR	E	82	60	27	55	5	8	2	1	1	0		4	7	4	7
	S	77	55	29	53	2	4	4	1	2	0		7	13	7	13
AkR	E	103	63	61	36	4	10	6	12	6	22	61	2	6	24	67
	S	103	77	75	22	4	15	2	3	5	7	32	3	14	10	46

* E = Extract; S = Saline.
† % of those weaned.
‡ % of those surviving 4 mo.

taneous incidence of leukemia remained low in the Rf strain, the unique feature of which is an extraordinary susceptibility to the induction of myeloid leukemia by ionizing irradiation(5,6).

Injection of newborn AkR mice with AkR extract greatly shortened the latency period of leukemia and increased the leukemia incidence as reported recently by Rudall *et al.* (7).

Immunogenetic Relationships. Table IV shows that adult Rf mice are sensitive to AkR cells, but "takes" occur in only about 10% of them when injected with large numbers of Ak leukemic cells. Adult mice of the other 5 strains proved resistant to AkR cells. Newborn mice of 2 strains (LAF_1 and C57BR), which proved resistant to the agent, were receptive to AkR cells. This indicates that histoincompatibility forces do not function in newborn mice.

Transplantation assays for the immunogenetic character of 13 leukemias developing in agent-injected mice (3 in Heb, 6 in Rf, 3 in DBA/1, and 1 in BALB/c) are summarized in Table V. All leukemias were grafted on mice of the strain of origin and on AkR mice. In 5 cases, further subpassages were made.

In the Heb strain, the 3 leukemic mice were 141-251 days old when sacrificed for transplantation assay. Two had marked generalized leukemia with thymic involvement, and proved to be immunogenetically related to the AkR strain. The grafted leukemias grew faster and in greater percentage in the strain of origin than in AkR. However, when a subpassage was made from the first-generation graft in an AkR mouse from Heb 17, leukemia developed in all of 5 AkR and in none of the strain of origin (Table V). It is unlikely that the leukemia was spontaneous in this donor AkR mouse because the latter was only 4 months old when sacrificed. The leukemia in Heb 20 certainly appeared "bivalent," since it "took" in all injected AkR and Heb mice. The third, presumably agent-induced leukemia (Heb 16), was microscopically an early case. Transplantation failed in the strain of origin but apparently succeeded in 2 AkR mice. One of these was probably transplanted, since it developed in a 4-month-old mouse (60 days after the graft) and was nonthymic. The second, being thymic and occurring late, may have been spontaneous.

Of the 6 transplanted leukemias arising in Rf mice, one grew in AkR only, 4 grew in both Rf and Ak, and one in neither strain. The one which appeared to be of the AkR type (Rf 55) could have been derived from Ak cells contaminating the extract. If so, it was latent in the Rf host for about 163 days. In general, the leukemias that were "bivalent" grew faster and in greater percentage in Rf than in AkR mice. Again, a subpassage from an AkR leukemia grew well in AkR. It is possible that this was spontaneous, since the donor mouse was nearly 5 months old when this subpassage was made. All 4 Rf mice grafted from this AkR died of infection 17 days after the graft, and had to be discounted. The 3 DBA/1 and 1 BALB/c leukemias grew only in the strain of origin. The very long induction period (428-435 days) of the 3 DBA/1 leukemias raises the question whether these cases were not spontaneous.

These data point to the necessity of carrying out transplantation assays on a much larger scale and with the use of proven highly inbred strains of mice with good genetic markers.

Mortality in agent-injected mice. Duran-Reynals has shown(8) that an oncogenic virus can cause a non-neoplastic disease in newborn animals. Mortality data (Table VI) were gathered to answer the question whether or not diseases other than leukemia might have been caused by the agent and whether some leukemias might have been missed at autopsy.

Table VI indicates that, in general, infant mortality was equally high among agent- and saline-injected animals. Indeed, in 5 strains mortality was slightly higher among the controls and in only 1 (Rf) was it distinctly higher among the extract-injected animals.

Leukemias did not occur until after the mice were 4 months old. In the interval between weaning and 4 months of age, mortality was higher among agent-injected mice in 4

strains, but in only 2 of these (DBA/1 and Rf) was there a definite increase of leukemia incidence in subsequent months. In 2 strains in which leukemia development was hastened and/or increased (AkR and Heb) mortality was higher among the saline-injected animals, even during the first 4 months. Mortality was caused by common infections and parasitisms.

In the 4 low-leukemia strains in which leukemia developed in extract-injected animals and was absent in saline-injected animals during the first 10 months of life, mortality from causes other than leukemia was invariably higher among the agent-injected animals, the difference being greatest in the 2 strains in which the incidence of leukemia was the highest (Rf and Heb). Since no systematic microscopic examination was made in animals grossly free of leukemia, it is not possible to state whether all those mice recorded to have died from causes other than leukemia were actually free from this disease, or whether the presence of the agent had lowered resistance to common infections, or otherwise caused death. The total mortality between the ages of 4 and 10 months appears to be as good an index of viral activity as the presence of grossly detectable leukemia.

Salivary gland and other tumors. Gross suggested that the virus carried in Ak mice is multipotent, capable of producing salivary gland and other tumors, or that Ak mice carry several oncogenic viruses. At autopsy, careful search failed to reveal neoplasms other than leukemias, except for two questionable salivary gland tumors in the first series. These findings do not support the view that the salivary gland tumors are caused by the same agent that induces leukemia.

Three salivary gland tumor strains (C3H) received from Dr. Gross were transplantable in C3H mice with variable success; none took in 100% of the animals and some regressed. They were not transplantable in Ak mice. This is in line with the work of others(9,10), indicating poor transplantability of these parotid tumors. The parotid glands of many C3H mice carrying grafted salivary gland tumors were examined microscopically. In none were incipient tumors noted, nor was there any evidence of other tumors or leukemias in hosts bearing such grafts.

Discussion. Three processes will now be considered by which leukemia can be caused: transplantation, induction, and transduction. Cellular grafts from spontaneous leukemias retain, as a rule, the genetic character of donor cells, whereas grafts from induced leukemias resemble closely or are identical with those of the host cells. Although this is the rule, it has also been reported that methylcholanthrene-produced leukemias differ among themselves immunogenetically(11). Bioassays indicate that leukemic cells are not present in young animals with genetic liability to leukemia(12). A primitive hemopoietic cell may become established in homologous newborn mice of diverse strains, and undergo leukemic transformation after a latency period (close to that of spontaneous leukemia). In this case, the leukemic cells are also expected to be of the donor type, and the process would be that of *transplantation* of precursor cells. Gross(13), and Woolley(14) working closely with Gross, have stated that their virus-induced leukemias were of the recipient type; this would indicate *induction.* Analysis of our transplantation results points to difficulties of interpretation, and suggests the possibility that some induced leukemias have the genetic character of both donor and recipient. Such a finding would be best explained by supposing that the transmitting agent is related to chromosomal matter (probably nucleoprotein) of the leukemic cells or their precursors which, like the DNA of certain microorganisms, can enter the reproductive matter (DNA genome) of other "infected" cells, either multiplying with them or causing perpetuation of their special genic characteristic by some other mechanism. Lederberg(15) named such processes *transduction.* Although it is doubtful that the transformations induced with lysogenic viruses (bacteriophage) are analogous with that of induced transformation of pneumococci, the term of Lederberg is accepted as a matter of convenience. In contrast, common infectious

viruses of mammals are best conceived as fully perpetuating themselves, the host merely contributing a biochemical environment, favorable to their multiplication.

The experiments here reported are inadequate to arrive at a definite conclusion on the nature of the agent which induces or hastens the development of leukemia in mice. Information is wanted on the effects of injection of extracts from low-leukemia strain mice,§ characterization of agents after passage through different strains, better correlation of immunologic and genetic relationships, and attempted inactivation of the agent with DNA-ase. Simple technical difficulties as encountered here in interpreting findings with this type of experiment will have to be overcome. How to certify inocula to be free from cells, and strains for adequate homozygosity? How long is the latency period of leukemias caused by transplantation of one or a few leukemic cells shortly after their origin? It is well known that neoplastic cells rapidly gain growth vigor in the course of passages, but it is not known when the neoplastic transformation occurs and how rapidly leukemic cells multiply in the original host. Inadequate inbreeding of the mouse strains, or lack of complete autonomy or genetic difference between strain and induced neoplastic cells could possibly account for non- or poor transplantability of some leukemias in the primary transfers. The strains used by us were adequately inbred for uniform success in transplantation of well-established leukemias, but inbreeding is a relative matter. Subtle immunogenetic changes are conceivable even in the course of neoplastic transformation in spontaneous leukemias, and antigenic simplification is a common occurrence in the course of transplantation.

A major problem requiring clarification is the type of leukemia induced in relation to the source of the agent. In the Ak strain, most spontaneous leukemias (80-90%) are thymic in origin. Is the thymus the sole or most sensitive organ attacked by this agent? In the present study all leukemias in agent-injected Ak mice were lymphoid with thymic involvement. In the other strains, however, many were generalized with involuted thymuses. Typing of leukemias requires special efforts not done systematically in the present study. The morphologic problems to be resolved include those of differentiation of advanced myeloid leukemoid reaction from leukemias, and of granulomatous lesions from neoplasms of monocytes (reticulum cells). Such a study calls for correlation of morphology with bioassays for malignant (autonomous) cells. Spontaneous leukemias in Ak mice are preventable by thymectomy(3). It follows that leukemia induction by this Ak agent would also be preventable by thymectomy. Attempts to recover the agent from nonthymic, spontaneous, and variously induced leukemias, and determination of the range of agents from different sources will have to be done. Since most nonthymic leukemias appear late in life, lifespan studies will be unavoidable. The Rf strain appears well suited for such studies because of its broader spectrum of sensitivity. In this strain, ionizing radiation causes thymic lymphomas (preventable by thymectomy)(5), myeloid (not so preventable), and reticulum-celled (monocytic) leukemias.

Three processes have been mentioned that could explain the development of leukemias following injection of tissue extracts from high-leukemia strain mice: transplantation, induction, and transduction. The first can now be excluded as a mere occasional complicating factor, the latter two are possibilities that merit equal consideration. Much has been said by others in favor of induction; the present work underlines the theory of transformation (transduction). Experiments suggesting it have been described and contradicted(3,16), and those here reported are likewise not conclusive. But the theory of transduction based on experiments on pneumococcal transformation would explain best the observed facts: the necessity of using newborn mice; the reported high sensitivity of strains which have some genetic relation to the Ak strain; the non-infectiousness of this

§ Latarjet (personal communication) failed to demonstrate an agent in his low-leukemia line 17.

agent under natural conditions; and the very long latency period, which is about that of the spontaneous disease. Latarjet(17) considered the possibility of the agent being similar to a prophage, but his experiments argue against this.

In general, causation of leukemia by virus can be conceived as: a) Stimulation of normal cells by intracellular specific or nonspecific "parasites." This would imply that the leukemias are virus-conditioned neoplasms. b) Induction of somatic mutation by a resident virus, proposed by Murphy(18) who named avian tumor viruses "mutagens." Mutation and abnormal differentiation, another possibility, imply an irreversible induction change in the cells, mutation being genetic and differentiation nongenetic in character. c) Recombination of chromosomes as occurs with transduction of genetic features of pneumococci and other microorganisms. Presently, all these possibilities(16) are useful as "working hypotheses."

Summary. 1. Tissue extracts from mice of the high-leukemia strain Ak injected into newborn mice greatly increased the leukemia incidence in Rf, DBA 1 and C_3Heb mice, and greatly hastened the development of leukemia in AkR mice. 2. Mortality without gross anatomic manifestation of leukemia was also enhanced in extract-injected mice of low-leukemia strains. 3. Although all leukemias in Ak mice were lymphoid with thymic involvement, many leukemias in the low-leukemia strains were of non-thymic types. 4. Transplantation assays of leukemias induced in the low-leukemia strains disclosed a heterogeneity in types, some being related immunogenetically with both host and donor strain. 5. Neither salivary gland nor other tumors were induced by the leukemia agent studied. 6. The available information suggests consideration of a process analogous with DNA-induced transformation (transduction) of genetic properties of microorganisms rather than to a natural type of viral infection.

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