INDUCTION OF BONE MARROW COLONY-STIMULATING ACTIVITY BY A FILTERABLE AGENT IN LEUKEMIC AND NORMAL MOUSE SERUM*

By ROGER FOSTER, Jr.,‡ M.D., DONALD METCALF,§ M.D., and ROSEMARY KIRCHMYER

(*From the Roswell Park Memorial Institute, Buffalo, New York 14203*)

(Received for publication 5 September 1967)

Sera from AKR mice with spontaneous lymphoid leukemia and Swiss mice with viral-induced leukemia have been shown to initiate and maintain proliferation of certain mouse bone marrow cells in a semisolid agar culture system (1, 3). The proliferating cells form colonies which originally contain granulocytic cells but later become composed entirely of mononuclear cells (2). Linear relationships exist between serum dose and the number and size of colonies developing from a standard number of bone marrow cells. A small proportion of sera from normal mice stimulate similar colony formation but the activity of these sera is weaker than that of leukemic sera. Sera from mice with breast, liver, or lung tumors or various spontaneous bacterial infections do not exhibit higher colony-stimulating activity than do sera from normal mice of the same age and strain (1).

In the course of this work it was observed that the intraperitoneal injection of sera from leukemic Swiss mice into normal adult Swiss mice resulted in the development of strong colony-stimulating activity in the sera of the recipient mice. The present report describes these findings and the properties of a transmissible agent present in some mouse sera which can induce elevated colony-stimulating activity in the sera of normal adult mice.

Materials and Methods

Mice.—Inbred DBA/1 and noninbred Swiss ICR/Ha mice were maintained in this institute. Noninbred germfree and conventional Swiss mice were obtained from the Charles River Breeding Laboratories (N. Wilmington, Mass.) and maintained in separate facilities in this institute. All Swiss mice used as recipients for serum injections were female Swiss ICR/Ha mice aged 3–5 months. Unless otherwise qualified all Swiss mice referred to in the

* This work was supported by United States Public Health Service Grants CA-08847 and CA-07745, the John A. Hartford Foundation, and the Anti Cancer Council of Victoria.

‡ Postdoctoral fellowship supported by United States Public Health Service Grant CA-501 6–10 from the National Institute of Health.

§ Present Address: Cancer Research Unit, Walter and Eliza Hall Institute, Melbourne, Australia.

results were Swiss ICR/Ha mice of the Roswell Park Memorial Institute (RPMI) colonies.

Leukemic Swiss ICR/Ha mice were supplied by Dr. Rita Buffett of Roswell Park Memorial Institute. These mice had been injected as newborns with passages 20–22 of a lymphoid leukemia-inducing virus whose isolation and properties have been described previously (4). When bled for serum testing, the mice had advanced lymphoid leukemia and were aged 5–8 months.

Bleeding.—Mice were bled from the orbital or axillary vessels under ether anesthesia using Pasteur pipettes. Clots were allowed to contract for 1–2 hr at room temperature and the sera stored at −20°C.

Bone Marrow Cultures.—Reagents were E_{2020} medium (Microbiological Associates, Bethesda, Md.); Earle's BSS 10 × stock, 100 ml; sodium bicarbonate 7.5%, 30 ml; MEM vitamins × 100, 20 ml; MEM amino acids × 50, 20 ml; MEM glutamine × 100, 10 ml; sodium pyruvate × 100, 10 ml; L-serine × 100, 10 ml; phenol red 0.5%, 4 ml; penicillin/streptomycin (5000 units of each per ml) 2 ml; distilled water, 94 ml; fetal calf serum (Baltimore Biological Laboratories, Baltimore, Md.) 100 ml. This mixture was filtered through a 0.45 μ Millipore membrane and stored at 4°C. Trypticase soy broth; 6 g trypticase soy broth (Baltimore Biological Laboratories) in 200 ml distilled water, autoclaved 20 min, and stored at 4°C. Bacto agar (Difco Laboratories, Detroit, Mich.) 0.6 g agar in 100 ml distilled water, boiled for 2 min, thoroughly dissolved and held at 40°C. Agar prepared immediately before use. Bone marrow–collecting fluid E_{2020} medium, 40 ml; trypticase soy broth, 10 ml; distilled water, 50 ml.

Bone marrow plugs from a single femur of three, 3- to 4-month-old DBA/1 mice were pooled in 5 ml of bone marrow–collecting fluid. A single cell suspension was prepared by vigorous pipetting and counted, usual cell counts were $4–6 \times 10^6$ nucleated cells per ml. Sera to be tested were pipetted into 35 mm plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.) in doses of 0.0125, 0.025, and 0.05 ml. Four parts of E_{2020} were mixed with one part of trypticase soy broth, and the bone marrow cell suspension added to give a concentration of 100,000 nucleated cells per ml. This cell suspension was held at 37°C, then mixed with an equal volume of 0.6% agar held at 40°C to give a final mixture containing 0.3% agar and 50,000 cells per ml. 1 ml aliquots of this mixture were pipetted into each Petri dish and mixed thoroughly with the serum. The dishes were left at room temperature (20°C) for 20 min to allow gelling, and then incubated at 37°C with a constant flow of 5% CO_2 in air. The incubator was humidified by passing the gas mixture through a gas disperser in the water bath at the base. Colony counts were performed routinely after 7 days of incubation, using a X 50 dissecting microscope. The criteria for colony scoring have been described previously (1, 3).

In the present experiments, as in previously reported work (1, 3), a linear relationship was found between serum dose and the number of colonies forming on each plate. Although all sera in the present study were tested at three dose levels, the data to be described refer only to colony stimulation by 0.025 ml dose levels, since this dose allowed good discrimination between active and inactive sera and was not subject to technical problems of surface drying, frequently encountered with high doses of mouse serum (3). A serum was scored as exhibiting colony-stimulating activity if more than five colonies were stimulated on a plate containing 0.025 ml of the mouse serum under test.

Ultracentrifugation.—Serum was spun in a model L-2 Beckman centrifuge (SW 39 L head) at 32,000 rpm (105,000 *g*) for 120 min at 5°C.

Ultraviolet Irradiation.—The UV source was a 15 watt low pressure Mercury vapor lamp 10 cm distant from the serum container. Serum was stirred continuously in a Petri dish during the 30 min exposure. As a control for the UV irradiation, vaccinia virus was subjected to a similar exposure and titrated on tissue culture cells.

Ether Treatment.—1 ml of serum was overlayed with 0.15 ml of ether and the mixture shaken continuously for 15 min at room temperature. The ether was bubbled off with nitro-

gen until no odor could be detected. Control serum was similarly treated except for the addition of ether.

Plasma Lactate Dehydrogenase (LDH) Estimations.—Plasma for enzyme determinations was collected in heparinized pipettes from the orbital venous plexus. Lactate dehydrogenase (LDH) was measured by the determination of the rate of oxidation of diphosphopyridine nucleotide (DPNH) in the presence of excess pyruvate at pH 7.5, according to the method of Riley (5). Enzyme activity was expressed in Wroblewski units per milliliter (International units = Wroblewski units/2).

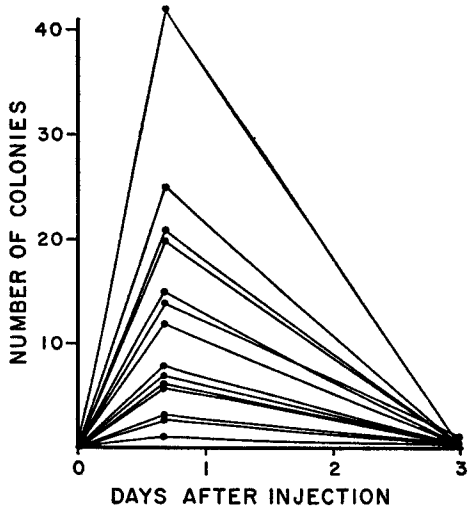

FIG. 1. Rise and fall in serum colony-stimulating activity following the injection of 0.1 ml of leukemic serum. Each line represents a single mouse.

RESULTS

Effects of Injection of Leukemic and Normal Serum.—

Individual normal Swiss mice were injected intraperitoneally with 0.1 ml of serum from 14 different leukemic Swiss mice whose sera had been shown to have strong colony-stimulating activity. The sera of recipient mice were tested for colony-stimulating activity at 16 hr and 3 days after injection. As is shown in Fig. 1, most sera of injected mice exhibited colony-stimulating activity at 16 hr but were inactive at 3 days. Colonies stimulated by the recipient's sera were essentially similar in total cell counts and cellular composition to those previously described as arising after stimulation by leukemic serum. However, the colonies frequently were more compact with tighter packing of colony cells than the loose colonies stimulated by leukemic AKR or Swiss serum (1, 3). No correspondence was observed between the level of colony-stimulating activity in the individual leukemic sera and the level of activity induced in the sera of recipient mice.

These observations were extended by testing leukemic and normal Swiss sera for their capacity to induce colony-stimulating activity in the sera of recipient mice at 16 hr after injection. 49 of 58 leukemic sera induced colony-stimulating activity in

the sera of recipient mice but only 10 of 80 sera from normal mice induced similar activity (Table I).

Additional tests were made of sera from a separate colony of germfree and conventional Swiss mice, obtained originally from the Charles River Breeding Laboratory. Sera from these mice were injected into normal Swiss mice, but in only one animal was colony–stimulating activity induced in the serum. As controls for these experiments, Swiss mice were sham injected or injected with 0.1 or 0.2 ml of buffered saline, fetal calf serum, or a variety of human sera. In no instance was colony–stimulating activity observed in the sera of the recipient mice at 16 hr after injection.

TABLE I

Induction of Colony-Stimulating Activity in the Serum of Normal Swiss Mice by the Intraperitoneal Injection of 0.1 ml of Leukemic or Normal Swiss Serum

Source of injected serum	No. of sera injected	No. of mice injected	Recipient mice with active sera*	Mean No. of colonies stimulated by recipient's sera
			%	
—	—	32	0	0.4
Leukemic Swiss	49	58	81	14.3
Normal Swiss	80	80	12	1.4
Germfree Normal Swiss (Charles River)	22	22	5	1.4
Conventional normal Swiss (Charles River)	23	23	0	0.9

* Stimulating the development of five or more colonies per plate. 0.025 ml of test serum used in plates containing 50,000 DBA/1 bone marrow cells.

Passage of Leukemic and Normal Serum.—

Individual serial passages were made of 27 different leukemic Swiss sera and 26 different normal Swiss sera in normal Swiss recipients, by injecting 0.1 ml of serum intraperitoneally into a single recipient at each passage. Recipient mice were bled 16 hr after injection and 0.1 ml of each serum reinjected into a new recipient after storage at −20°C for intervals varying from 4 hr to 6 wk. Due to the restricted numbers of available animals, not all passage lines were maintained after passage 2 but no selection of passage lines was made. The results (Table II) indicated that the capacity of leukemic serum to induce elevated colony–stimulating activity in the sera of the recipient mice could be passaged successfully. Some increase in inducing potency of sera occurred with continued passage as shown by the increased numbers of colonies stimulated by the sera of recipient mice.

However, variation was encountered in individual passages, some having weak activity (e.g. the 10 leukemic passage lines at passage 9) which recovered on repassage. Within individual passage lines, similar variability was encountered and in no case did every recipient of a single passaged leukemic serum respond by developing strong serum colony–stimulating activity. At the end of these individual passage experi-

ments, all passaged leukemic serum was pooled and maintained by repeated passage through normal mice as "pooled passaged serum" for use in subsequent experiments.

The agent inducing elevated serum colony-stimulating activity has not proved easy to maintain using continuous 16-hr passages. With a number of pooled passaged serum lines, infectivity was lost or was greatly decreased after 5–10 passages. On several occasions, continued passage of this material resulted in the reacquisition of potency. It is possible that infectivity decreases when serum is stored at −20°C, or the passage interval of 16 hr may be too short to allow adequate replication of the inducing agent in all recipient mice.

TABLE II

Effect of Serial Passage on Capacity of Leukemic and Normal Swiss Sera to Induce Colony-Stimulating Activity in the Sera of Recipient Mice

Passage No.	Leukemic serum		Normal serum	
	No. of mice with active sera/No. injected*	Mean No. of colonies stimulated by recipient's sera	No. of mice with actve sera/No. injected*	Mean No. of colonies stimulated by recipient's sera
1	25/27	21.0	1/26	0.7
2	25/27	28.0	4/26	2.5
3	10/10	32.6	7/21	17.0
4	10/10	43.8	10/21	15.1
5	8/10	32.7	8/10	21.7
6	9/10	24.1	3/10	4.8
7	8/10	18.6	5/10	11.7
8	9/10	34.5	6/10	8.6
9	3/10	6.3	4/9	9.7
10	8/10	19.8	—	—

* Stimulating the development of five or more colonies from 50,000 DBA/1 bone marrow cells, using 0.025 ml serum.

In agreement with earlier data only one of the recipients of the 26 different normal sera developed colony-stimulating activity in the serum at the first passage but the potency of the sera increased on passage, so that by passage 5 the recipients of sera from 8 of 10 passaged normal serum lines developed strong serum colony-stimulating activity. Colonies stimulated by passaged normal serum appeared to be identical with those stimulated by passaged leukemic serum.

Physical Properties of Inducing Agent in Passaged Leukemic Serum.—Pooled passaged leukemic serum (passages 10–15) was treated in various ways (Table III) and then tested directly on bone marrow cells for its capacity to stimulate colony formation and, by injection into mice, for its capacity to induce colony-stimulating activity in the sera of the recipients. Injection of large groups of mice with the same pool of passaged serum showed that individual mice did not

develop a uniform level of serum colony–stimulating activity, possibly explaining some of the variation encountered in the earlier individual passage lines.

The properties of the colony-stimulating factor in passaged serum were identical with those described previously for the colony–stimulating factor in the serum of leukemic Swiss mice (3). The colony stimulating factor was filterable through 0.45 and 0.22 μ Millipore membranes and withstood heating at 60°C for 30 min and UV irradiation. With ether treatment, the results were variable, some batches of pooled sera retaining colony–stimulating activity

TABLE III

Effect of Various Pretreatments on Capacity of Passaged Leukemic Serum to Induce Elevated Serum Colony–Stimulating Activity in Recipient Mice

Pretreatment	Direct test	Induction test	
	Mean No. of colonies stimulated by treated passaged serum	No. of recipient mice with active serum* No. injected	Mean No. of colonies stimulated by recipient's serum
—	48.0	5/6	22.2
Filtered 0.45 μ	49.0	8/9	30.3
Filtered 0.22 μ	24.0	3/3	37.7
Heated 60°C 30 min	62.5	0/5	1.0
Sham-ether treated	51.0	4/4	25.3
Ether treated	0.0	0/5	0.0
Sham UV Irradiated	39.0	4/4	36.8
UV Irradiated	48.5	0/5	0.0
Centrifugation 105,000 *g* 2 hr‡			
Upper supernate	50.0	2/5	7.2
Lower supernate	61.0	4/5	13.2
Pellet	0.5	5/5	23.6

* Stimulating the development of five or more colonies.

‡ Different passaged serum pool.

while others lost their activity. From the appearance of the culture plates it seemed possible that toxic products of the ether inactivation procedure sometimes killed the bone marrow cells soon after plating out, preventing the possibility of colony formation. Centrifugation of passaged serum at 105,000 *g* for 2 hr did not appear to reduce the colony–stimulating activity of the serum in the upper half of the centrifuge tube and the serum pellet when resuspended in pretested inactive normal serum, did not exhibit colony–stimulating activity.

The properties of the inducing agent in passaged leukemic serum differed sharply from those of the colony–stimulating factor itself. The inducing agent was filterable through 0.45 and 0.22 μ Millipore membranes, but was destroyed by heating at 60°C for 30 min, ether treatment, and UV irradiation. Centrifugation of whole serum resulted in a decrease in inducing activity in the upper half

of the supernatant serum and sedimentation into the serum pellet of strong inducing activity (pellets were resuspended in tissue culture fluid before injection into test mice).

Effects of Passaged Agent from Leukemic Serum on Recipient Mice.—

Groups of normal Swiss mice were injected with 0.1 ml of pooled passaged leukemic serum and examined at intervals from 4 hr to 6 days after injection. No consistent

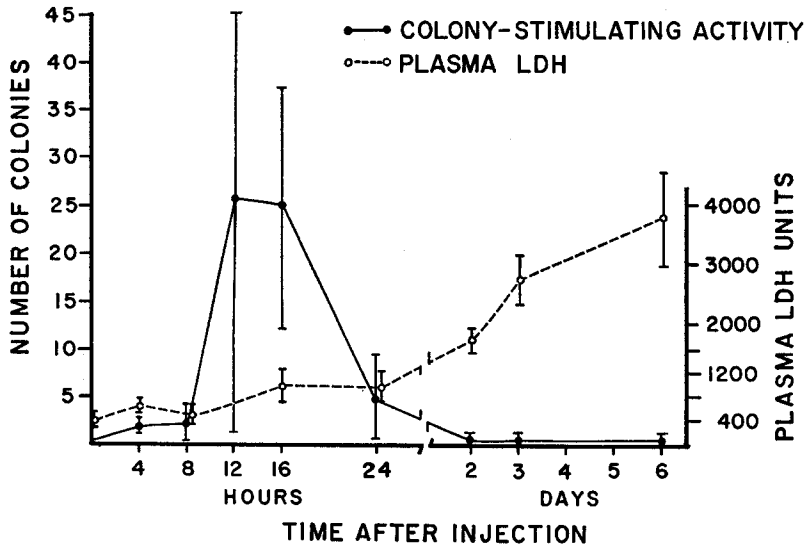

FIG. 2. Effect of 0.1 ml of passaged leukemic serum on serum colony-stimulating activity and plasma LDH levels. Each point is mean value for five mice. Vertical bars are standard deviations of values.

changes were observed in lymphoid organ weights, hematocrit levels, or lymphocyte and polymorphonuclear levels in the blood.

Serum colony-stimulating activity began rising at 8 hr and reached maximum levels between 12 and 16 hr after injection (Fig. 2). After this time serum activity fell rapidly, suggesting a serum half-life for the colony-stimulating factor of less than 4 hr. After 24 hr no colony-stimulating activity was detectable in the sera of the majority of injected mice.

When the sera of injected mice were assayed at intervals after injection for infectivity (i.e. their capacity to induce elevated serum colony-stimulating activity in new recipient mice), a peak of infectivity was observed between 8 and 16 hr after injection (Fig. 3). After this time infectivity appeared to fall, but unlike the situation with the level of colony-stimulating factor, the serum remained infective throughout the observation period of 6 days.

Parallel observations on plasma LDH levels in the mice injected with passaged leukemic serum indicated that LDH levels began rising 16 hr after injection, and

continued to rise throughout the 6 day interval, reaching levels of 3500–5600 units/ml (Fig. 2).

Effect of LDH–Elevating Virus and Other Viruses on Serum Colony–Stimulating Activity.—

In view of the similarity in passageability of the present agent from leukemic serum with that of the LDH–elevating virus, and the rise in plasma LDH levels induced by the passaged leukemic serum agent, assays were made of serum colony–stimulating activity following infection of Swiss mice with LDH–elevating virus

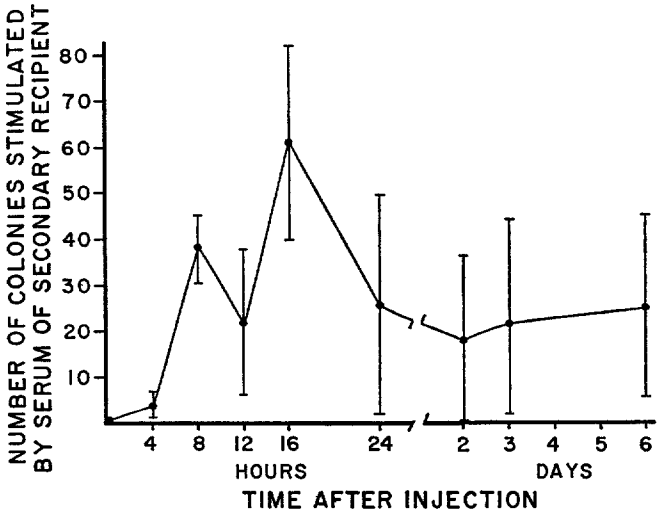

FIG. 3. Infectivity of recipient's serum after the injection of 0.1 ml of passaged leukemic serum assayed by injection of serum from recipient into a secondary recipient. Each point is mean value for five mice. Vertical bars are standard deviations of values.

obtained from Dr. Vernon Riley of the Sloan-Kettering Institute, N. Y. Observations were also made on the effect of a number of other viruses on both serum colony–stimulating activity and plasma LDH levels.

Of the various viruses tested (Table IV) both the LDH–elevating virus and Newcastle disease virus (N.D.V.) induced high levels of serum colony–stimulating activity in recipient mice 16 hr after infection. Small rises in serum colony–stimulating activity, of doubtful significance, were observed in some recipients injected with Sendai virus and Sinbis virus but no response was observed to the injection of PR8, adeno-7, herpes simplex, and "human leukovirus" concentrates prepared from cultures of Burkitt lymphoma cells. Rises in plasma LDH levels were observed following injection of LDH–elevating virus and N.D.V. but not following the injection of the other viruses. The negative results following the injection of viruses other than the LDH–elevating virus and N.D.V. should be regarded with caution as with none of these viruses was an attempt made to verify that an active infection had occurred in the injected mice.

The time courses of changes in levels of serum colony-stimulating activity and plasma LDH following the intraperitoneal injection of Swiss mice with the LDH-elevating virus are shown in Fig. 4. It will be noted that serum colony-stimulating activity peaked at 16 hr as in the response to passaged leukemic serum and that the time course of the rise in plasma LDH levels also was similar to that observed in the response to passaged leukemic serum. Titration of LDH-elevating virus revealed that serum colony-stimulating activity was elevated at 16 hr only when more than 10^5 infectious doses (as assayed by LDH elevation) were injected. Serum activity was elevated by a dose of 0.1 ml of a 1:20,000 dilution of the original infective mouse

TABLE IV

Effect of Various Viruses on Serum Colony-Stimulating Activity and Plasma LDH

Virus injected	Source	Titer	No. mice with active serum/No. injected*	Mean No. of colonies stimulated by recipient's serum	Mean plasma LDH units per ml‡
LDH-elevating virus	Mouse	ID_{50} $10^{9.3}$/ml	35/41	24.0	3500
Newcastle disease virus	Egg	ID_{50} $10^{7.8}$/ml	10/19	11.0	1440
Sendai	Egg	HA 1:10,000	1/8	1.9	864
Sinbis	Monkey kidney T.C.	ID_{50} $10^{8.0}$/ml	2/8	1.6	495
PR 8	Egg	HA 1:1024	0/8	0	786
Adeno 7	Monkey kidney T.C.	—	0/8	0	550
Herpes simplex	Rabbit embryo T.C.	ID_{50} $10^{7.2}$/ml	0/8	0.1	650
Human leukovirus	Burkitt lymphoma T.C.	8.5×10^9 particles/ml	0/8	0.4	640

* Serum tested at 16 hr. Positive sera stimulated development of five or more colonies.

‡ Plasma tested on day 4 after infection.

plasma supplied by Dr. Riley but not by 0.1 ml of a 1:200,000 dilution of this plasma. Titration of the agent from passaged leukemic serum indicated a loss of capacity to induce elevation of serum colony-stimulating activity when the serum was diluted beyond 1:100.

Observations were made on the relationship between the development of elevated serum colony-stimulating activity and of elevation of plasma LDH levels in mice injected with normal serum. Nine such normal sera were injected into recipient mice and these sera were serially passaged four times. In most cases a rise in serum colony-stimulating activity was associated with a rise in plasma LDH levels (Table V) although many examples were noted where plasma LDH levels rose in the absence of any elevation of serum colony-stimulating activity.

Further evidence of a correlation between the incidence of the LDH-elevating virus and the agent elevating serum colony-stimulating activity was obtained in a study of the response of normal Swiss mice to injections of sera from Charles River Swiss mice. Swiss mice were injected with 28 different germfree or conventional

Charles River Swiss sera. In no case was serum colony-stimulating activity elevated and in contrast to the response of mice to the injection of RPMI Swiss sera, only one recipient of the 28 different Charles River Swiss sera developed a slight elevation of serum LDH.

Rechallenge of Mice Injected with Inducing Agent.—

Groups of Swiss mice were injected with 0.1 ml of either leukemic Swiss serum or passaged leukemic serum. The sera of these mice were tested at 16 hr postinjection

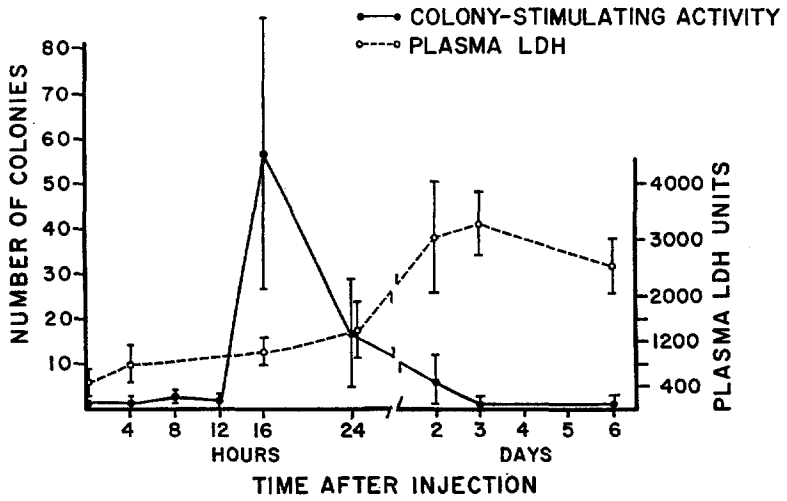

FIG. 4. Effect of $10^{9.3}$ ID_{50} of LDH-elevating virus on serum colony-stimulating activity and plasma LDH levels. Each point is mean value of five mice. Vertical bars are standard deviations of values.

to confirm that they exhibited colony-stimulating activity. At intervals from 7 days to 3 wk later, these mice were retested to confirm that their sera no longer exhibited colony-stimulating activity, then reinjected with 0.1 ml of passaged leukemic serum. 16 hr after rechallenge the sera of these mice were tested for colony-stimulating activity. Of 35 mice rechallenged, none developed colony-stimulating activity in the serum following the challenge injection of active passaged serum (Table VI). 10 mice were preinjected with 10 separate normal mouse sera and tests showed a failure to develop serum colony-stimulating activity. When these mice were rechallenged 3 wk later with passaged leukemic serum only 4 of the 10 developed colony-stimulating activity, suggesting that some degree of resistance had developed subsequent to the injection of some of the normal sera, despite a uniform failure to respond initially to the normal sera. Saline-injected control mice responded to challenge injection with passaged leukemic serum by developing high levels of colony-stimulating activity.

In a further series of experiments mice preinjected with 0.1 ml of a 1:20 or 1:20,000 dilution of mouse plasma containing 10^{10} ID_{50} of LDH virus failed to develop elevated

TABLE V

Relation between Elevation of Serum Colony-Stimulating Activity and Elevation of Plasma LDH in Mice Injected with Passaged Normal Serum

Passage line No.	Passage 1		Passage 2		Passage 3		Passage 4	
	Serum C.S.A.*	Plasma LDH	Serum C.S.A.	Plasma LDH	Serum C.S.A.	Plasma LDH	Serum C.S. A.	Plasma LDH
1	—	380	—	684	—	536	—	2748
2	—	2940	—	2672	+	2788	+	3740
3	—	496	—	3320	+	2940	—	4016
4	—	N.T.	—	1260	+	532	—	2864
5	—	684	—	380	—	1104	—	2404
6	—	1868	+	380	—	2672	—	3056
7	—	2788	+	4240	—	N.T.	+	3552
8	—	992	—	840	—	2596	+	3360
9	—	3056	—	N.T.	—	N.T.	—	3092

N.T., not tested.

* C.S.A., serum colony-stimulating activity. Positive sera stimulated the formation of five or more colonies per plate at the 0.025 ml dose level.

TABLE VI

Resistance to Rechallenge of Mice Preinjected with Either Passaged Leukemic Serum or LDH Virus

Rechallenged with	Preinjected with	No. mice with active serum/ No. injected	Mean No. colonies stimulated by serum
Passaged leukemic serum	—	20/22	30.5
	Passaged leukemic serum	0/35	0.4
	Normal mouse serum	4/10	9.9
	LDH virus 1:20	0/15	0.7
LDH virus 1:20	—	38/46	25.0
	LDH virus 1:20	0/14	1.6
	LDH virus 1:20,000	1/6	2.2
	Passaged leukemic serum	4/20	3.4
	Passaged normal serum	1/9	4.1

levels of serum colony-stimulating activity when 0.1 ml of a 1:20 dilution of plasma containing LDH virus was reinjected 3 wk later. Finally, groups of mice previously injected with either pooled, passaged leukemic serum, or LDH virus were cross-challenged 3 wk later with LDH virus or passaged leukemic serum. As is shown in Table VI, cross-resistance or a cross-refractory state was exhibited between both agents and in neither situation did rechallenged mice develop elevated serum colony-stimulating activity.

DISCUSSION

The agent in leukemic serum which induces elevated levels of colony-stimulating activity in the sera of recipient mice has properties consistent with those of a virus, being passageable, filterable, sedimentable, and heat-, ether-, and UV-labile. The incidence of infection with this virus, or viral titers in the serum, appears to be higher in leukemic than in normal Swiss mice, and some strains like the Charles River Swiss mice may not carry the agent.

It would seem improbable that the agent inducing colony-stimulating activity within 16 hr after injection is a murine leukemia virus in view of the very rapid replication of the agent and its persistence at high titers following serial 16-hr passage in adult mice. The stationary or eclipse phase upon passage of many of the murine viral leukemias is reported as 3 days at a minimum (6–8).

The capacity for rapid replication in the host animal and the induction of elevated levels of plasma LDH enzyme in infected animals suggests that the virus may be similar to, or identical with, the LDH-elevating virus described by Riley et al. (9). LDH virus was found to cause an elevation of serum colony-stimulating activity in Swiss mice identical with that caused by the passaged leukemic serum agent, provided that the original inoculum contained more than 10^5 infectious doses of LDH-elevating virus. Further, mice injected with the passaged leukemic serum agent were refractory to subsequent injection of LDH-elevating virus, and vice versa. This latter phenomenon may not represent cross-immunity in the classical sense, rather the development of a cross-reacting state of refractoriness to the induction of elevated levels of serum colony-stimulating activity. Mice injected 7 days earlier with passaged serum agent did not respond to a second injection of passaged serum agent but tests of the infectivity of serum of mice injected 7 days previously indicated a persistent viremia, making an immune status unlikely as a basis for the acquired refractory state. Rowson et al. have found that serum-neutralizing activity against the LDH agent of Riley is demonstrable in the late stages but not early after infection and further that in chronically infected mice a high proportion of the circulating virus resists neutralization (10). While it would seem most probable that the production of colony-stimulating activity is part of a response to the initial intense viremia it may be that colony-stimulating activity in the serum does rise following a second injection and the apparent refractory state may be due to the development of an inhibitory factor in the serum masking the expression of the colony-stimulating factor.

Although there was not complete parallelism in all cases between elevation of plasma LDH and serum colony-stimulating activity, the results support in general the hypothesis that the passaged leukemic serum agent is LDH-elevating virus, but that LDH-elevating virus can only induce an elevation of colony-stimulating activity if inoculated in high titers. It is still possible that preparations of passaged serum agent and LDH-elevating virus each contain

two viruses and that the two viruses have similar properties and similar rapid rates of replication. This possibility seems unlikely but more precise methods of physical separation will be required to eliminate the possible presence of two similar viruses in these virus preparations.

The capacity of LDH-elevating virus to elevate serum colony-stimulating activity may not be unique, for even in the limited series of tests of other viruses in the present study, Newcastle disease virus was found also to stimulate elevation of colony-stimulating activity. Many viruses and perhaps other infectious agents may share a capacity to induce the development of elevated levels of colony-stimulating activity in the serum of the host animals. This would be in agreement with survey data on colony-stimulating activity in mouse and human sera, where serum activity has been found to be very low in germfree mice (11), to be high in mice from colonies with a high endemic rate of disease caused by pneumonia virus of mice, and to be elevated in humans in the acute stages of certain viral and protozoal infections.[1] The biological function of the colony-stimulating factor in the intact animal remains uncertain but its properties and occurrence suggest that it may be a normal humoral factor regulating leukopoiesis and that serum levels of the factor are elevated in acute infections when white cell changes are occurring as part of the host response to infection.

If the present passaged serum agent is LDH-elevating virus it would appear that leukemic mice develop and sustain a high level viremia, possibly as a result of recrudescence of an infection acquired earlier in life.

Infection of mice with LDH-elevating virus or passaged leukemic serum agent provides a simple method for obtaining large volumes of mouse serum with high colony-stimulating activity, and this source of colony-stimulating factor is being used in further physicochemical studies on the nature of the colony-stimulating factor.

SUMMARY

1. Leukemic Swiss mice of ICR/Ha strain which had been injected at birth with a lymphoid-leukemia-inducing virus preparation yielded sera which produced elevations of serum colony-stimulating activity within 16 hr and significant plasma-LDH-enzyme elevation at 4 days when injected intraperitoneally into normal ICR/Ha Swiss mice. Colony-stimulating activity was assayed in vitro by the stimulation of hemopoietic colony formation by DBA/1 bone marrow cells.

2. The inducing agent in leukemic serum was passageable, filterable, sedimentable, and heat-, ether-, and UV-labile.

3. A similar agent was recovered from normal Swiss serum after blind serial passages through normal mice.

[1] Foster, R., Jr., and D. Metcalf. Manuscript in preparation.

4. LDH elevating virus induced a similar elevation of serum colony–stimulating activity when injected at high titers, and cross-resistance was demonstrated between LDH virus and the passaged leukemic serum agent.

We are indebted to Dr. Vernon Riley of the Sloan Kettering Institute for providing stocks of LDH-elevating virus and for technical advice on the measurement of plasma LDH levels.

BIBLIOGRAPHY

1. Robinson, W., D. Metcalf, and T. R. Bradley. 1967. Stimulation by leukemic and normal mouse sera of colony formation in vitro by mouse bone marrow cells. *J. Cellular Physiol.* **69**:83.
2. Metcalf, D., T. R. Bradley, and W. Robinson. 1967. Analysis of colonies developing in vitro from mouse bone marrow cells stimulated by kidney feeder layers or leukemic serum. *J. Cellular Physiol.* **69**:93.
3. Metcalf, D., and R. Foster. 1967. Bone marrow colony stimulating activity of serum from mice with viral induced leukemia. *J. Natl. Cancer Inst.* **39**:1235.
4. Buffett, R. F., J. T. Grace, Jr., and E. A. Mirand. 1964. Properties of a lymphocytic leukemia agent isolated from Ha/ICR Swiss mice. *Proc. Soc. Exptl. Biol. Med.* **116:** 293.
5. Riley, V. 1963. Enzymatic determination of transmissable replicating factors associated with mouse tumors. *Ann. N. Y. Acad. Sci.* **100**:762.
6. de Harven, E., and C. Friend. 1960. Origin of the viremia in murine leukemia. *Natl. Cancer Inst. Monograph.* **4**:291.
7. Spencer, H. J. 1963. Rapid intracerebral passage and recovery of moloney leukemia virus from mouse brain tissue. *J. Natl. Cancer Inst.* **30**:313.
8. Rauscher, F. J. and B. V. Allen. 1964. Growth curve of a murine leukemia virus in mice. *J. Nat. Cancer Inst.* **32**:269.
9. Riley, V., F. Lilly, E. Huerto, and D. Bardell. 1960. *Science.* **132**:545.
10. Rowson, K. E. K., B. W. J. Mahy, and M. Bendinelli. 1966. Riley virus neutralizing activity in the plasma of infected mice with persistent viremia. *Virology.* **28**:775.
11. Metcalf, D., R. Foster, and M. Pollard. 1967. Colony stimulating activity of serum from germ free normal and leukemic mice. *J. Cellular Physiol.* **70**:131.