

SERUM-MEDIATED LEUKEMIA CELL DESTRUCTION IN AKR MICE

ROLE OF COMPLEMENT IN THE PHENOMENON

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The importance of the AKR mouse strain in leukemia research is widely recognized. Leukemia develops in 80–90% of AKR mice with a peak incidence at 8 mo of age and this characteristic has been maintained since the strain was derived by Jacob Furth in 1935 (1). Study of AKR mice allowed Furth and his coworkers (2) to discover the essential role of the thymus in leukemia development and led Gross (3) to his discovery of the murine leukemia virus. Newer immunological and virological techniques capable of tracing virus and viral antigens in AKR mice are now providing the basis for defining the genetic determinants of virus transmission and viral gene expression (4, 5).

One of the widely held views of experimental tumor immunology some years back was that the AKR mouse (before as well as after leukemia development) was immunologically tolerant of the various antigens associated with its indigenous leukemia virus (6). This conclusion was based on what appeared at the time to be substantial evidence. From birth to death, AKR mice possess the spectrum of cellular (7), viral (8), and soluble antigens (9) that accompany infection with wild-type Gross leukemia virus. Characteristic C type virus particles could be visualized budding from normal and neoplastic cells and RNA leukemogenic virus could be isolated throughout the life-span of the mouse. Antibody against cellular and soluble antigens could not be detected in the blood of AKR mice at any age (10), nor could transplantation resistance to syngeneic tumor cells be clearly demonstrated (11). We now know from the work of Oldstone et al. (12) and Mellors et al. (13) with murine leukemia virus (MuLV)¹ that the presence of virus and viral antigens throughout life, and the failure to detect antibody in the serum of infected mice, do not exclude the occurrence of a specific immune response. Specific antibody can be demonstrated in the glomeruli of infected mice, trapped there presumably as antigen-antibody complexes. The absence of readily demonstrable serum antibody in AKR mice can be ascribed to the relative rates of antigen and antibody production, synthesis and release of virus and viral antigens evidently surpassing the production of antibody. But despite the presence of an immune response to at least some MuLV-associated antigens in AKR mice, it is not an effective one in terms of virus suppression or inhibiting the growth of leukemic cells, so far as we can tell.

¹ *Abbreviations used in this paper:* CNV, composite numerical value; CVF, cobra venom factor; MuLV, murine leukemia virus.

The work to be recorded here had its origin in the studies of Graff et al. (14) on the effect of interferon in AKR mice with spontaneous leukemia. Marked reduction in the size of leukemic lymph nodes and spleens occurred within 18–24 h after interferon injection. Morphological evidence for destruction of leukemia cells could be seen at 2½ h by light microscopy and as soon as 30 min by electron microscopy (15). The cytotoxicity appeared to be selective for leukemia cells, normal lymphocytes apparently remaining intact and uninjured. Two observations suggested that the effect is not due to a direct cytotoxic action of interferon on leukemia cells. First, the viability and metabolic activity of AKR leukemia cells in vitro is not influenced by added interferon. Second, the serum of interferon-treated AKR mice with leukemia (at a time when interferon has been cleared from the blood) is itself capable of causing striking reduction in leukemic lymph nodes and spleens of other AKR leukemic mice (16).

We have now found that normal serum from a variety of species, including the mouse, mimics the effect of interferon on AKR leukemia. The evidence obtained, so far, points to a component of the complement system as the anti-leukemic factor in normal serum.

Materials and Methods

Infusion Technique.—Intravenous infusion in the mouse is readily accomplished through the tail vein, providing that immobilization of the tail is maintained. This can be done without anesthesia by the use of a conical wire cage attached to a plastic “paddle shaped” base (Fig. 1). The mouse is placed in the wire cage and the tail is immobilized by fixing it in position on the “paddle handle” with one-half inch masking tape at the distal end. Motion is further limited by a small strip of tape across the base of the tail. To cannulate the tail vein, the vein must be dilated and this is done either before placing the mouse in the cage (by dipping the tail into warm water) or after restraining the animal (by applying a warm gauze pad over the vein). A 27 gauge needle attached to the 10 ml plastic syringe by silicone rubber tubing is used to cannulate the vein and is fixed in position by tape coming up from under the “paddle handle.” If more than one infusion of the same animal is planned, the needle should be inserted in the most distal portion of the vein and care should be taken when removing the needle to prevent trauma to the vein. Rate of infusion is controlled by a Varioperex peristaltic pump (LKB, Bromma 1, Sweden), with input delivery set at 1 ml/15 min. Material to be tested was diluted to 6 ml in pyrogen-free normal saline and, at this rate, infusion was completed in 90 min. It is possible to extend this period to as much as 7.5 h (without undue harassment to the mouse) by means of the variable speed available on the pump.

Serum.—Female Swiss mice (obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass.) and mice of inbred strains (from Jackson Memorial Laboratories, Bar Harbor, Me. or from our colonies) aged 3–8 mo were anesthetized with ether and bled from the tail. Normal and C4-deficient guinea pigs (supplied by Dr. I. Green, Division of Immunology, National Institutes of Health, Bethesda, Md.) were bled by cardiac puncture while under light ether anesthesia. Horse, rabbit, and human sera were obtained by venepuncture. Blood was collected in pyrogen-free bottles and was allowed to clot for 1–2 h at room temperature. Cells were removed by centrifugation at $650 \times g$ at 4°C and the serum was aliquoted in volumes adequate for administration to several mice and stored at –78°C. Samples were thawed just before use and were not refrozen. Individual mice were infused with 1.5 ml mouse serum, 0.5 ml human serum, or 0.75 ml guinea pig serum or horse serum; the sera were diluted to 6 ml with pyrogen-free normal saline immediately before infusion.

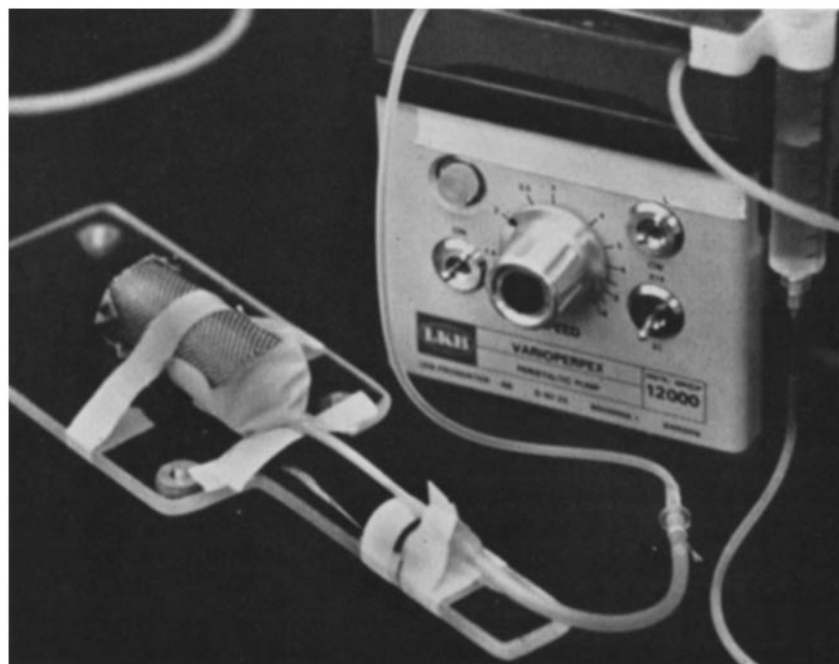


FIG. 1. Procedure for tail vein infusion in the mouse. Wire mesh cone attached to plastic paddle restrains mouse for intravenous infusion. Rate of infusion controlled by peristaltic pump (see text for details).

Evaluation of Leukemic Mice.—A large colony of retired AKR/JAX female breeders served as a source of leukemic mice. The mice were examined weekly for the development of lymphadenopathy and splenomegaly. The degree of enlargement of the (a) inguinal, (b) cervical, and (c) mesenteric lymph nodes, and (d) spleen were scored from 1⁺ to 4⁺ on the basis of palpation. The sum of these four scores is called the composite numerical value (CNV) and was used to evaluate changes in the leukemic mouse brought about by treatment. Thus, the percentage reduction in CNV is equal to $100 - (\text{CNV at 24 h postinfusion} / \text{CNV before infusion} \times 100)$. The subjective limitations of this evaluation are such that a reduction of less than 20% in the size of the lymph nodes and spleen was not considered significant.

Thymic enlargement was suspected when evidence of respiratory distress, bowing of the chest, and characteristic hunching was observed. This parameter could not be accurately evaluated, but was noted as an indication of greater disease involvement than was numerically recorded. Animals were examined frequently during the infusion and postinfusion period, and CNV estimated at 24 and 48 h after completing the infusion.

Interferon.—The preparation of tissue culture interferon has been described in previous reports (14, 16). Briefly, mouse cells (L929) grown in suspension culture were infected with Newcastle disease virus. After an 18 h incubation period, cold ethanol was added to the cell-free culture medium and the precipitate dialyzed against 2% acetic acid. The resulting material was frozen, lyophilized, and taken up with heat-inactivated horse serum. Antiviral activity was assayed on L929 monolayers challenged with vesicular stomatitis virus. Mice were infused with 2,500–5,000 U of interferon in 6 ml of normal saline.

Endotoxin.—*Escherichia coli* endotoxin, 0127:B8 (no. 3123-10) was obtained from Difco

Laboratories, Detroit, Mich. Stock preparations of 1,000 $\mu\text{g}/\text{ml}$ were dissolved in normal saline and stored at -20°C . A series of preliminary experiments showed that the infusion of endotoxin at 10 μg (in 6 ml saline) caused greater than 50% reduction of CNV in leukemic AKR mice.

Cobra Venom Factor (CVF) (see ref. 17, 18).—Lyophilized CVF was purchased from Cordis Laboratories, Miami, Fla. It was reconstituted in cold distilled water to a concentration of 100 U/ml, subaliquoted and stored at -78°C . For in vitro de complementation, serum was diluted to 6 ml with normal saline (see above) and incubated with 10 U of CVF/ml of serum at 37°C for 30 min. For in vivo de complementation, AKR leukemic mice were injected i.v. with 50 U CVF 18 h before infusion with serum, interferon, or endotoxin.

Complement Components (19, 20).—Individual complement components from human and guinea pig were purchased from Cordis Laboratories. They were reconstituted with cold distilled water, aliquoted, and stored at -78°C . Each of the components was tested by infusing leukemic mice with 500 CH50 U diluted to 6 ml in normal saline. Individual components are reported by Cordis to contain less than 10 CH50 U/ml of contamination by other complement components.

RESULTS

Infusion of Normal Mouse Serum.—AKR mice with advanced leukemia were infused with serum from random-bred Swiss mice or from mice of seven different inbred strains. The strains selected represent those with the complete spectrum of complement components in their serum (Swiss, C57BL/6, B10D2 new, SJL/J) and those lacking the C5 component of the complement system (AKR, A, B10D2 old, DBA/2).

Fig. 2 shows that serum from C5⁺ mouse strains can cause a striking reduction in the size of lymph nodes and spleen in leukemic mice, whereas the serum of C5-deficient mice was generally ineffective. The antileukemic activity of serum from Swiss mice was generally greater than serum from C5⁺ inbred strains. Infusion of normal saline under comparable conditions produced no change in leukemic mice.

Fig. 3 illustrates the effect of an infusion of Swiss mouse serum on a leukemic AKR mouse. Within 24 h there is a marked reduction in the size of leukemic lymph nodes and spleen. Microscopic evidence of leukemia cell destruction can be seen as early as 1½ h postinfusion (Fig. 4) and by 3 h phagocytosis of degenerating leukemia cells is evident. Destruction of leukemia cells was also observed in blood smears from animals with circulating leukemia cells. Within 1–2 h after infusion of Swiss mouse serum, cytoplasmic vacuolization and nuclear pyknosis and fragmentation were observed in peripheral leukemia cells, and this was followed by a marked drop in the peripheral white cell count.

Infusion of Heterologous Serum.—Serum from normal guinea pigs, horses, or humans causes leukemia cell destruction and shrinkage of leukemic lymph nodes and spleen when infused in AKR leukemic mice (Fig. 5). Serum from C4-deficient guinea pigs was comparable to serum from normal guinea pigs in antileukemic activity. Serum from normal or C6-deficient rabbits (obtained from Rancho de Conejo, Vista, Calif.) proved too toxic to evaluate even at levels of 0.1 ml/mouse. Removal of the naturally occurring heteroantibodies

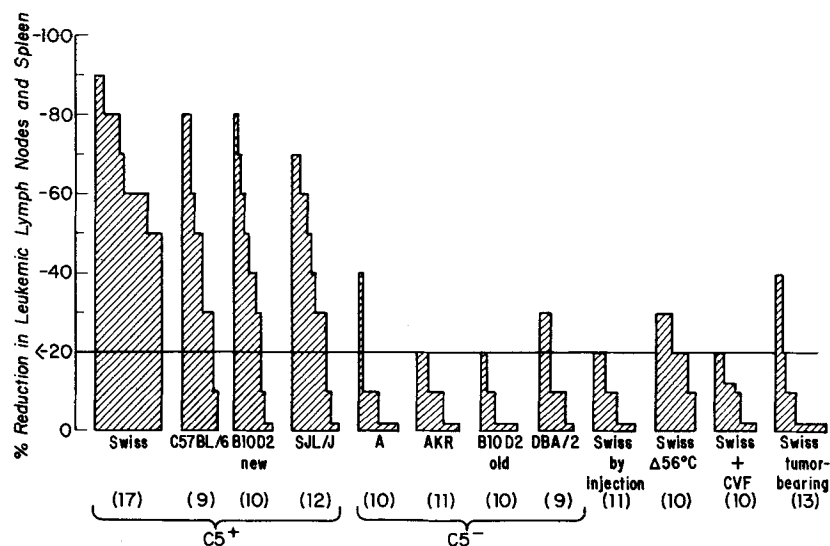


FIG. 2. AKR leukemic mice infused with normal serum from C5⁺ and C5⁻ mouse strains. Mice evaluated at 24 h postinfusion (see text for details). Reduction >20% considered significant. Number in parenthesis indicates number of leukemic mice tested. Results: C5⁺ mouse serum causes reduction in leukemic lymph nodes and spleens. C5⁻ mouse serum is inactive. The antileukemic factor in Swiss mouse serum is (a) not demonstrable by injection (in contrast to infusion), (b) heat labile, (c) inactivated by CVF in vitro, and (d) reduced in tumor-bearing mice.

in rabbit serum by absorption with mouse tissues (as in the preparation of complement for cytotoxic tests with mouse cells [21]) removed the acute toxicity for leukemic mice. Such absorbed serum demonstrated good antileukemic activity.

Injection vs. Infusion of Serum.—We did not observe any antileukemic effect when the mouse, horse, or human serum was given as a single injection, rather than by infusion (Fig. 2). Injection of undiluted serum (i.v. or i.p.) or serum diluted to 6 ml with normal saline (i.p.) in amounts that were highly effective by infusion gave no reduction of leukemic lymph nodes or spleen.

Effect of Heat Inactivation.—The antileukemic property of Swiss serum was abolished by preincubating the serum at 56°C for 30 min, a procedure known to inactivate certain components (including C5) of the complement system (Fig. 2). Similarly, the activity of horse and human serum in leukemic mice is also markedly reduced after heat-inactivation (Fig. 5).

Toxicity and Subsequent Fate of Infused Leukemic Mice.—Infusion of saline, C5-deficient normal mouse serum, or heat-inactivated homologous or heterologous serum was tolerated with little toxicity. Mice receiving C5⁺ serum showed varying degrees of lethargy, ruffled coat, and decreased food intake during the 12–24 h postinfusion period. In general, signs of toxicity paralleled the observed

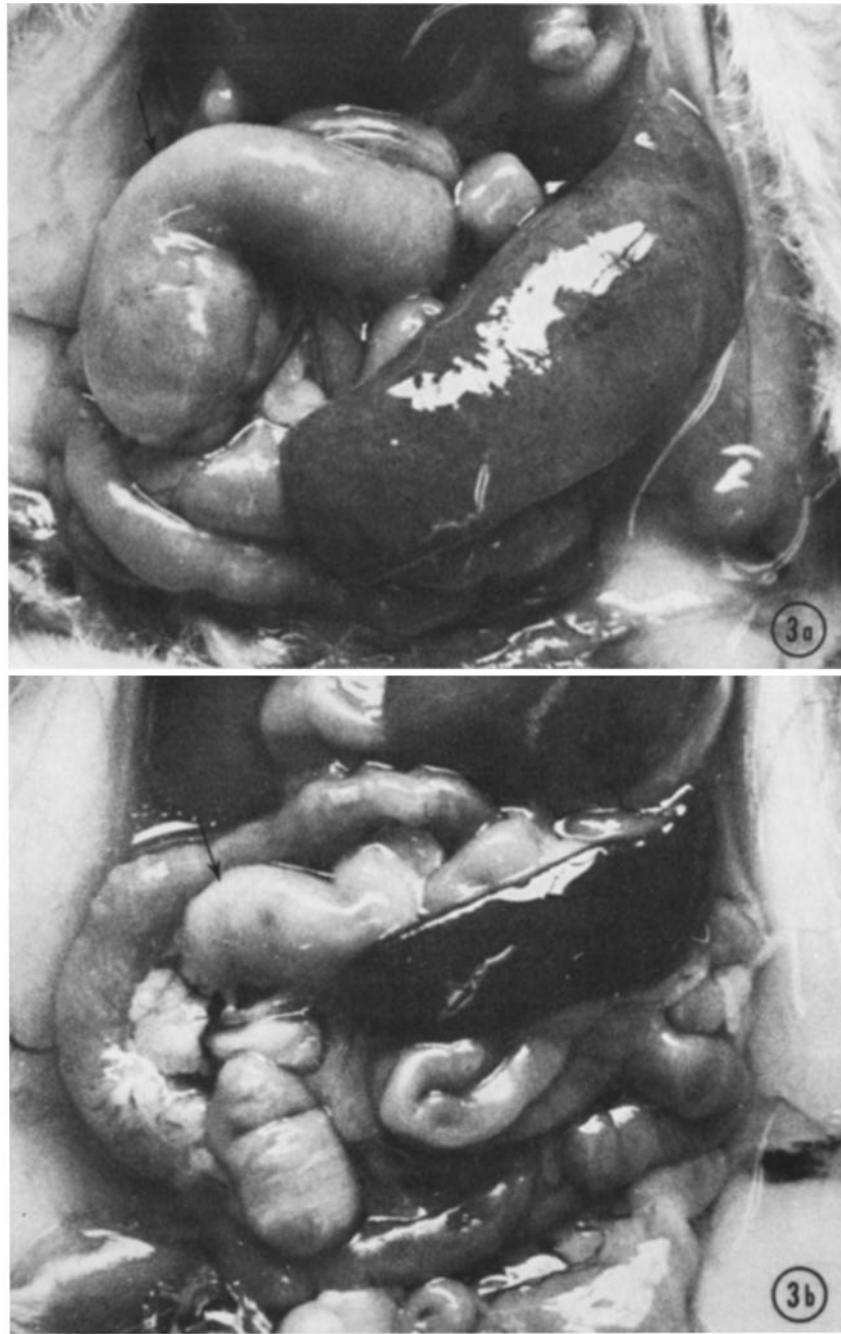


FIG. 3. AKR mice with spontaneous leukemia. (a) Untreated leukemic mouse with massively enlarged mesenteric lymph nodes (arrow) and spleen. (b) A leukemic mouse with comparably advanced disease that had been infused with serum from normal Swiss mice 24 h previously; marked reduction in size of mesenteric lymph nodes (arrow) and spleen.

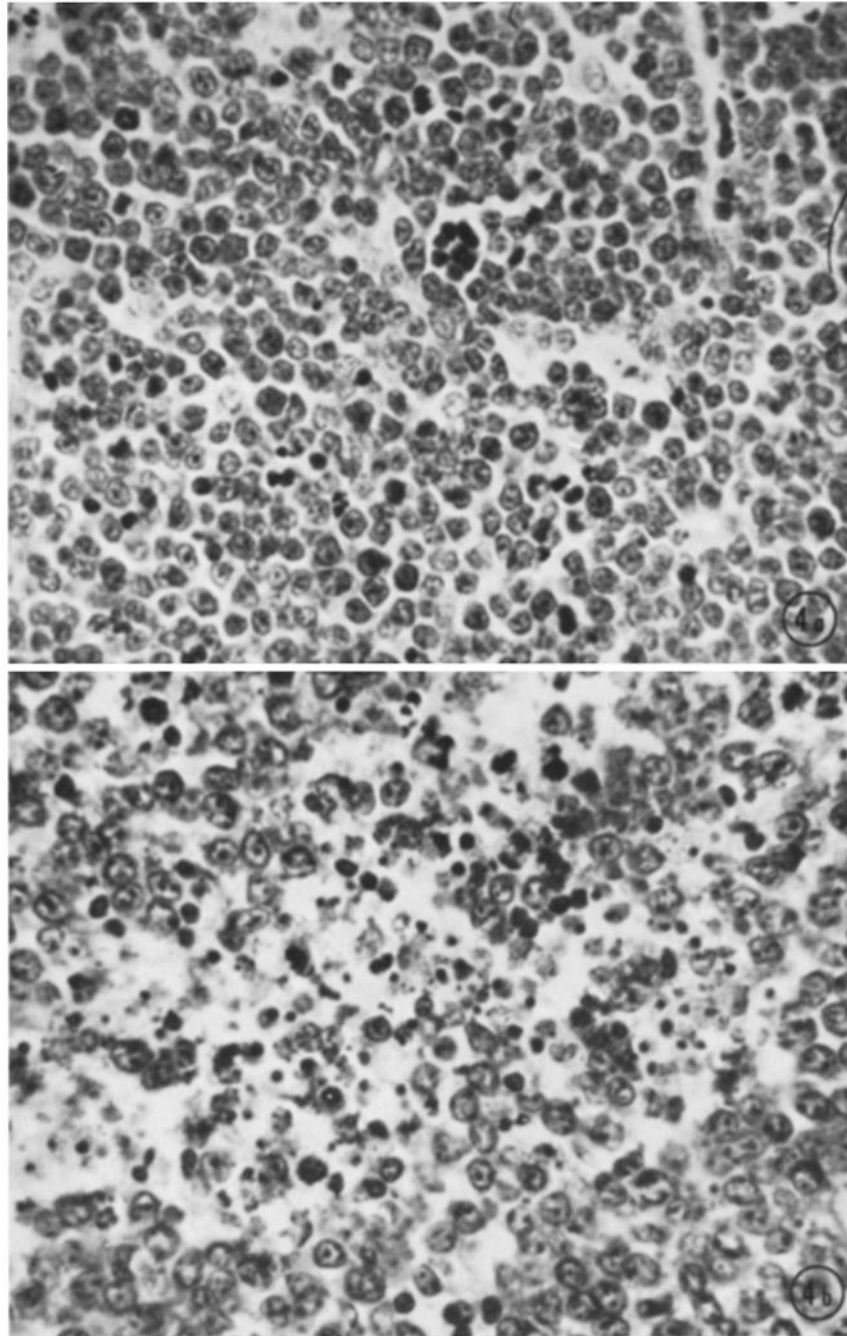


FIG. 4. Mesenteric lymph nodes of leukemic AKR mice. (a) Untreated leukemic mouse. Lymph node replaced by proliferating leukemic lymphoblasts. (b) Leukemic mouse $1\frac{1}{2}$ h after infusion with serum from normal Swiss mice. Lymph node in initial phase of leukemia cell destruction. Magnified $400\times$.

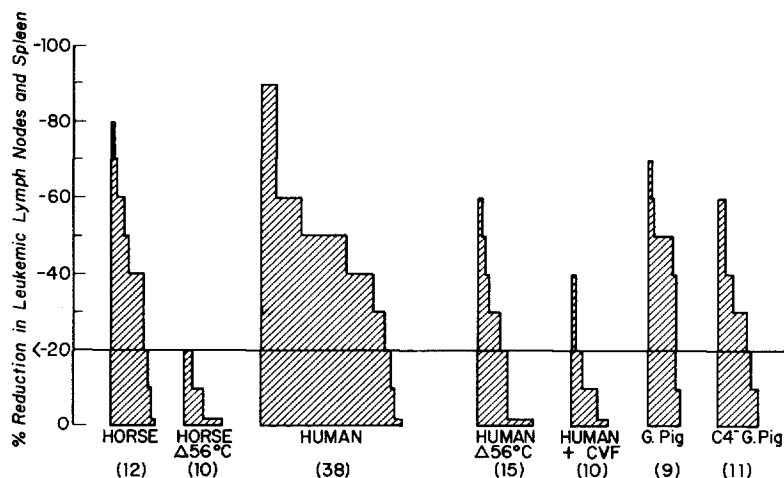


FIG. 5. AKR leukemic mice infused with normal serum from heterologous species (see Fig. 2 and text for details). Results: Horse, human, and guinea pig serum cause reduction in leukemic lymph nodes and spleen. The antileukemic factor is (a) heat labile (horse and human serum) and (b) inactivated by CVF in vitro (human serum).

reduction of leukemic lymph nodes and spleen. C5⁺ mouse serum produced less toxic effects than heterologous serum. Within 24–36 h, all toxicity associated with serum infusion disappeared. By the fifth day postinfusion, a resurgence of disease was observed in most leukemic mice.

Infusion of Serum from Tumor-Bearing Mice.—Serum was collected from Swiss mice that had received transplants of Ehrlich ascites or Tupper liver tumor (i.p. or s.c.) 7–8 days previously. In contrast to serum from normal Swiss mice, infusion of serum from tumor-bearing Swiss mice caused no reduction in the size of leukemic lymph nodes or spleen (Fig. 2).

Effect of Cobra Venom Factor.—Incubating Swiss mouse serum or human serum with CVF in vitro before infusion into leukemic AKR mice abolished the antileukemic activity of these sera (Figs. 2 and 5). Similarly, leukemia cell destruction did not occur if leukemic animals were injected with CVF 18–20 h before the infusion of Swiss mouse serum.

Effect of Interferon.—As in previous studies (16), infusion of interferon caused a marked reduction in the size of lymph nodes and spleen (Fig. 6). Preincubating the interferon preparation with CVF before infusion did not alter its antileukemic activity. If on the other hand, CVF was given to the leukemic mouse 18–20 h before interferon, then interferon caused no reduction of leukemic lymph nodes or spleens at 24 h postinfusion (Fig. 6). At 48 h, however, interferon-mediated destruction of leukemia cells had occurred, showing that the action of interferon had been delayed but not abolished by CVF.

Effect of Endotoxin.—Infusion of 10 μg of endotoxin caused marked reduction in the size of lymph nodes and spleen (Fig. 6). Characteristic endotoxin toxicity

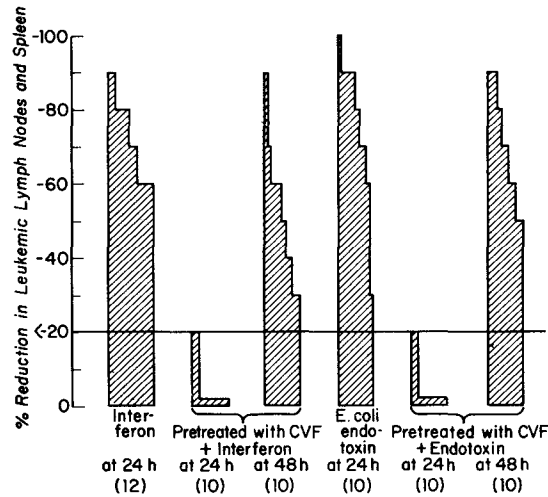


FIG. 6. AKR leukemic mice infused with interferon and endotoxin (see Fig. 2 and text for details). Results: Interferon (5,000 IU) and *E. coli* endotoxin (10 μ g) cause reduction in leukemic lymph nodes and spleens within 24 h. Pretreating leukemic mice with CVF (50 U) delays the antileukemic effect of interferon and endotoxin by 24 h.

accompanied the leukemia cell destruction—diarrhea, prostration, and weight loss (2–3 g over a 24–36 h period). These toxic manifestations disappeared within 2–3 days after endotoxin treatment. As with interferon (see above), pretreating the mouse with CVF delayed, but did not abolish, the antileukemic effect of endotoxin (Fig. 6). Preincubating endotoxin with CVF in vitro or heating endotoxin to 56°C for 30 min did not alter its antileukemic activity. Endotoxin toxicity was markedly reduced in leukemic mice pretreated with CVF.

Antileukemic Activity of Individual-Complement Components.—Nine complement components from guinea pig serum and nine from human serum were tested individually by infusion in AKR leukemic mice (Fig. 7). C5 of both guinea pig and human origins stood out as giving consistent reduction of leukemic nodes and spleen. The other complement components were generally ineffective. The antileukemic activity of the C5 preparations showed a dose dependence over the range of 200 U/mouse to 2,000 U/mouse. Infusion of C5 (in comparison to injection) was necessary to demonstrate the C5 effect. Heating the C5 to 56°C for 30 min abolished its antileukemic activity.

DISCUSSION

The infusion of normal serum from homologous or heterologous sources leads to the rapid destruction of leukemia cells. The fact that this is observed in mice with spontaneously developing leukemia adds considerable significance to the observation. Prolonged infusion of the serum is critical in demonstrating

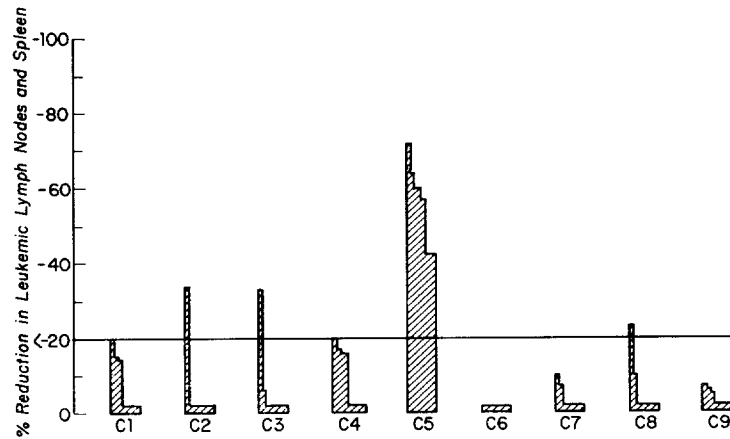


FIG. 7. AKR leukemic mice infused with complement components from guinea pig serum and from human serum. Each of the complement components was tested in four AKR leukemic mice; the findings for individual components from the two species were identical and were therefore plotted together (see Fig. 2 and text for details). Results: C5 is the antileukemic complement component in normal guinea pig and human serum.

the effect. Comparable amounts of serum given by injection rather than by infusion were inactive, a likely reason why the phenomenon has not been observed before.

Present evidence points to a component of the complement system as mediating the effect. The findings underlying this conclusion are as follows:

(a) CVF abolishes the antileukemic effect of normal serum. CVF depletes complement by activating C3, thereby setting off the step-by-step consumption of $C3 \rightarrow C9$ terminous (17). The ability of CVF to cancel the serum-mediated lysis of leukemia cells was shown in two ways: preincubating serum with CVF before infusion or pretreating leukemic mice with CVF before infusing active serum.

(b) Leukemia cell destruction was induced by serum from mouse strains possessing all components of the complement system but not by serum from mice with genetically determined deficiency of C5. So far, eight strains have been tested and the correlation with the C5 trait has been consistent.

(c) Fractions rich in C5 from both human and guinea pig serum showed antileukemic activity, whereas other C fractions (C1-4 and C6-9) were without effect. As AKR mice are genetically C5-deficient (22-24), it may not be surprising that the antileukemic-complement component in normal serum is C5. Apparently C4 is not generally limiting in the leukemic mouse; C4-deficient guinea pig serum was essentially equal to nondeficient guinea pig serum in antileukemic activity.

How does C5 bring about leukemia cell destruction in AKR mice? For the sake of discussion, we can put forward three possibilities:

(a) C5 has a direct effect on leukemia cells by providing the missing complement

link which allows cytotoxic antibody produced by the host to cause cell lysis. According to this view, progressive growth of leukemia cells in AKR mice would not be due to lack of specific cytotoxic antibody but lack of a secondary factor (C5) involved in leukemia cell destruction. Past studies by Kidd (25) and by our group (26) have indicated that complement, not specific antibody, may be the limiting factor in determining the effectiveness of immune reactions to tumors in the mouse. We have observed that *H-2* incompatible ascites sarcomas may grow progressively in highly immunized mice (in this case from a C5⁺ strain) even though the tumor cells could be shown to have attached cytotoxic alloantibody; injection of guinea pig serum as a complement source brought about rapid rejection of these far-advanced ascites tumors. Apparently the mouse is limited in its capacity to generate sufficient C when called upon to reject large numbers of tumor cells. Thus, complement deficiency (whether relative or absolute) can be viewed as another mechanism whereby tumor cells escape the consequences of their antigenicity. In this regard, reduced levels of complement have been found in the serum of tumor-bearing mice (27, 28). This provides the most likely explanation for our observation that such sera lack antileukemic activity in AKR mice.

(b) *C5 reacts at some site other than the leukemia cell surface, e.g., with antigen-antibody complexes in the blood or kidney, thus causing the release of lymphokins, steroids, etc., that themselves mediate leukemia cell destruction.*

(c) *The antileukemic effect of C5 is independent of a preexisting immune response, revealing another mechanism for specific killing of leukemia cells.*

Studies are under way to test these various possibilities. Perhaps the key question is whether AKR mice form specific cytotoxic antibodies to autogenous leukemia cells. We are looking for attached immunoglobulins on the surface of leukemia cells and for cytotoxic antibody in serum of AKR mice, especially after leukemia cell destruction induced by serum, interferon or endotoxin.

Our studies with CVF reveal that complement appears to be involved, also, in the antileukemic activity of interferon and endotoxin. These agents cause a remarkable reduction in leukemic lymph nodes and spleen 24 h after their administration. In leukemic mice, pretreated with CVF, no antileukemic effect is seen at 24 h after interferon or endotoxin infusion; by 48 h, however, a pronounced reduction in leukemic nodes and spleen has occurred. The 24 h delay is presumably the time required for complement levels to return to normal. Although complement is known to be involved in other actions of endotoxin, such as in the Schwartzman phenomenon (18), no such role has been suggested for complement in interferon action. It would be important to determine whether other activities of interferon, e.g., antiviral and antileukemic activity *in vitro* (29), can be modified by CVF or other inhibitors of the complement system. Two pathways of complement activation have now been well established, the "classical" pathway (via C1, 4, and 2) (19) or through a group of serum proteins comprising the properdin or "alternate" pathway (30, 31). In both, C5 plays a key roll. If complement is involved in the antileukemic activity

of interferon and endotoxin, how does this take place in AKR mice which are genetically deficient in C5? Is there a C5 shunt mechanism? Or might exogenous interferon or the interferon (or other factors) induced by endotoxin substitute in some way for C5?

The phenomenon of leukemia cell destruction mediated by normal serum is not restricted to the mouse. We have found that cats with lymphosarcoma, undergo dramatic resolution of their disease when infused with normal cat serum. Large lymphomatous masses, characteristically of the chest and abdomen, essentially disappear after 2 wk of serum infusion on alternate days. 8 out of 10 cats so treated have shown this response.² The critical role of complement in this antileukemic effect in cats needs to be determined.

These studies in mice and cats suggest that serum-mediated destruction of leukemia cells may occur in man, and that the occasional remission after blood transfusion in patients with leukemia can be explained on this basis. Low complement levels have been observed in the serum of leukemic patients (32); this may account for our finding (unpublished) that serum from 16 of 20 patients with untreated leukemia failed to cause leukemia cell destruction in AKR mice. In view of these several leads, a closer look at the complement system in patients with leukemia and other cancers is clearly needed.

SUMMARY

AKR mice with spontaneous leukemia were infused with normal serum from a variety of species. Leukemia cell destruction was produced by serum from strains of mice possessing the full spectrum of complement components, but not by serum from strains with a genetically determined deficiency of C5. Serum from guinea pigs, horses, and humans also causes destruction of leukemia cells. The antileukemic factor in normal serum was heat labile (56°C for 35 min) and could be inactivated by cobra venom factor (CVF). Tests of individual complement factors from guinea pig serum and from human serum suggest that C5 is the antileukemic complement component in normal serum. Evidence was obtained that complement also plays a role in the antileukemic effect of interferon and endotoxin.

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