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Plasma-Mediated Leukemia Cell Destruction: Concentration and Purification of the Antileukemia Factor[1] (39779)

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Introduction. Selective destruction of neoplastic cells has been observed in leukemic mice (1), cats, and dogs (2) following infusion of normal serum, plasma, or whole blood. In AKR mice, the antileukemia activity of serum is mediated by the C5 component of the complement system. Similar involvement of the complement system has not been proved in the antileukemic effects noted in cats or dogs. Attempts to reproduce the phenomenon *in vitro* by incubating AKR leukemia cells with serum, plasma, or C5 have failed, and this has limited analysis of the factors involved in leukemia cell destruction. However, regardless of mechanisms, these observations of antileukemia activity of normal blood in three species suggest that this approach can be put to use in cancer therapy and that the occasional, but well-documented, remissions in leukemia patients following transfusions of normal blood or plasma (3, 4) may represent the counterpart of this reaction in man.

We have recently noted that heparinized plasma has significantly greater antileukemia activity (ALA) than serum in leukemic AKR mice (5). Heparinized plasma (HP), stored at 4° overnight, develops a precipitate which adsorbs or carries with it the ALA, thus allowing its concentration and purification. We have also found that ALA can be isolated by adsorption onto a calcium phosphate gel. Using these methods, plasma ALA can be concentrated rapidly and with little expense, resulting in preparations that can be administered repeatedly because of low protein content.

Materials and methods. Retired AKR/J female breeders serve as our source of leukemic mice and are examined weekly for lymphadenopathy and splenomegaly. For quantitative evaluation of leukemic mice, the following method has been developed and found to yield reproducible results (1). The degree of leukemic enlargement of the (a) inguinal, (b) cervical, and (c) mesenteric lymph nodes, and (d) spleen are scored from 0 to 4^+ on the basis of palpation. The sum of these four scores is used to evaluate changes (in terms of percentage reduction) as a consequence of treatment. The subjective limitations of this evaluation are such that a reduction of less than 20% in the size of the nodes and spleen is not considered significant (1).

Groups of five AKR leukemic mice were infused with samples to be tested and were evaluated at 24 hr for percentage reduction of lymph nodes and spleen. Intravenous infusion through the tail vein was performed by methods described previously (1). In our initial studies, we found it necessary to use the method of infusion in order to observe ALA, since injection of the same quantity of serum intravenously (as a single bolus) had no effect. Eluates from HP precipitates or calcium phosphate gel appear to differ in this regard, since ALA has been observed following rapid intravenous injection. However, the reduction of leukemic lymph nodes and spleen is significantly greater following infusion of active material, and this method has therefore been used to evaluate ALA.

Heparinized blood was obtained from 4- to 6-month-old CD-1 ($C5^+$) female mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) by retro-orbital puncture, from pregnant sows by jugular bleed-

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ing, and from human volunteers by venipuncture.

Results. Table I illustrates the comparative ALA of mouse serum, whole blood, heparinized plasma, and citrated plasma. These studies demonstrated that HP has greater activity than serum and that citrated plasma is inactive. During these experiments we observed that mouse, pig, and human HP held at 4° overnight develops a fibrin-like precipitate. Elution of this precipitate with 0.15 *M* NaCl for 10 min at room temperature results in a supernatant possessing ALA (Table II). ALA is stable when stored at 4° or −78° as the precipitate; it is less stable as the eluate.

As a similar precipitate forms in HP from AKR ($C5^-$) mice (from which ALA cannot be eluted), it was felt that the ALA factor from $C5^+$ plasma might be loosely adsorbed to the precipitate as a heparin complex. In reviewing the literature on heparin action, the reports of Burstein and Scholnick (8) and Srinivasan *et al.* (9) describe the complexing of heparin and serum lipoproteins with cations. This complex formation is reversible and is dependent upon the ionic strength of the cation and its binding characteristics. As the Ca^{2+}–heparin–lipoprotein complex was reported to be totally dissociable (in contrast to complexes formed with Mn^{2+} and Mg^{2+}), an attempt was made to adsorb the ALA onto a calcium phosphate gel. This method of purification, which has been used widely in enzyme isolation (10), probably depends on adsorption as well as ion exchange. Our initial experience has been that ALA can be isolated from HP by gel adsorption and that gel elution and readsorption of ALA is dependent on ionic

TABLE I. COMPARATIVE ANTILEUKEMIA ACTIVITY OF SERUM, WHOLE BLOOD, HEPARINIZED PLASMA, AND CITRATED PLASMA IN AKR LEUKEMIC MICE.[a]

Material tested (by infusion)[b] from CD-1 mouse donors ($C5^+$)	Number of animals	Average reduction (%)	Reduction in individual leukemic mice (%)
Serum, 0.5 ml	20	24	10 (12 mice) 30, 30, 30, 40, 40, 50, 70, 70
Serum, 1.5 ml	17	62	40, 40, 43, 45, 57, 58, 58, 58, 60, 60, 62, 70, 70, 80, 80, 80, 90
Heparinized plasma (10 U/ml), 0.5 ml (tested immediately)	10	52	14, 33, 39, 46, 56, 62, 66, 66, 66, 75
Heparinized plasma (10 U/ml), 0.5 ml (frozen for 4 days, 78°)	10	10	0, 0, 6, 8, 12, 13, 15, 16, 17, 20
Citrated plasma (ACD)[c], 0.5 ml (tested immediately)	10	<10	0, 0, 0, 0, 6, 6, 8, 8, 12, 14
Whole blood, 1.0 ml (tested immediately)	6	49	25, 35, 40, 58, 65, 70
Heparin, 400 U	8	<10	0, 0, 0, 4, 6, 8, 10, 11
0.15 *M* NaCl	7	<10	0, 0, 0, 0, 10, 10, 10

[a] For evaluation of AKR leukemic mice and calculation of percentage of reduction, see text. Dr. I. M. Mountain, Biostatistician at Sloan-Kettering Institute, has calculated that for groups of five AKR leukemic mice, the upper confidence limits for an average decrease of 18% are $P < 0.05$ and for 23% decrease are $P < 0.01$.

[b] For infusion, material to be tested was diluted to a total volume of 6 ml in 0.15 *M* NaCl [see (1)].

[c] ACD constituents per milliliter of blood: citric acid, 0.4 mg; sodium citrate, 3.32 mg; sodium phosphate, 0.28 mg; and dextrose, 3.22 mg.

TABLE II. ANTILEUKEMIA ACTIVITY OF ELUATES FROM HEPARINIZED PLASMA (HP) PRECIPITATES.

Eluate of HP precipitate from	Protein content (Lowry; mg/ml)	Number of animals	Average reduction (%)	Reduction in individual leukemic mice (%)
AKR ($C5^-$)	7.8	10	<10	<10 (10 mice)
CD-1 ($C5^+$)	5.0	23	40	22, 22, 25, 28, 28, 28, 30, 33, 33, 36, 38, 39, 39, 40, 40, 42, 45, 47, 57, 58, 59, 59, 82
Pig ($C5^+$)	9.7	8	36	0, 23, 25, 32, 43, 50, 50, 64
Human ($C5^+$)[a]	5.5	8	50	28, 33, 39, 40, 50, 57, 70, 82

[a] DiLuzio *et al.* (6, 7) have described an opsonic factor isolated from human heparinized plasma precipitates. The relationship between his opsonic factor and ALA is being investigated.

strength (as described for Ca^{2+}–heparin–lipoprotein complexes). Utilizing these characteristics of ALA, it has been possible to develop the purification procedures outlined in Table III, which provide an additional method for concentration and purification of ALA from mouse, pig, or human plasma. Adsorption of ALA to the calcium phosphate gel is specific, resulting in the elimination of 82% of the plasma proteins

TABLE III. PURIFICATION OF ANTILEUKEMIA ACTIVITY FROM HEPARINIZED PLASMA OF CD-1 ($C5^+$) MICE BY CALCIUM PHOSPHATE GEL ADSORPTION.

		Protein content (Lowry)	Average percentage reduction [individual response]	Concentration index[a]
Heparinized plasma		72 mg/ml (1.0 ml/infusion)	48 (43, 50, 50, 50, 47)	1
0.1 ml of calcium phosphate gel/ml of plasma 30 min at room temp (R.T.)				
Centrifuge 500*g* ×	10 min			
Supernate discard	Gel: Resuspend in 0.4 ml of cold 0.02 *M* $CaCl_2$/ml of plasma	supernate = 59.55 mg/ml (1.0 ml/infusion)	<10 (not significant) [0, 30, 16, 10, -12]	
Centrifuge				
Supernate discard	Gel: Repeat cold $CaCl_2$ wash	1st wash = 3.41 mg/ml (1.0 ml/infusion) 2nd wash = no detectable protein	<10 [6, 8, 0, 0, 20] (not determined)	
Supernate discard	Gel: Elute with 0.2 ml of 0.15 *M* NaCl/ml of plasma, mix for 10 min @ R.T.			
Centrifuge				
Gel discard	Supernate = E_1	E_1 = 2.03 mg/ml (0.2 ml/infusion)	48 [55, 50, 47, 43, 43]	180
	Dilute with 3 vol of distilled water, add 0.1 ml of gel/ml, mix 30 min @ R.T.			
Centrifuge				
Supernate discard	Gel: Elute with 0.15 *M* NaCl as above			
Centrifuge				
Gel discard	Supernate = E_2	E_2 = 0.400 mg/ml (0.2 ml/infusion)	45 [33, 50, 55, 46, 40]	960

[a] In monitoring steps in the concentration and purification of ALA, we have employed the following formula to determine a concentration index (CI).

$$\frac{\text{Average reduction by HP (\%)} \times \text{milligrams of protein administered}}{\text{Average reduction by ALA test material (\%)} \times \text{milligrams of protein administered}}$$

Average reduction calculated from five AKR leukemic mice per assay.

following the first gel adsorption. Subsequent washing with cold, low ionic strength calcium chloride does not remove active material from the gel, but serves to eliminate some trapped proteins. Elution with 0.15 *M* NaCl releases the ALA. Changing the ionic strength of the eluate allows readsorption onto a fresh gel, thus providing further purification.

In view of our evidence that complement mediates the ALA of normal plasma in AKR leukemic mice (1), emphasis is being placed on C analysis of ALA isolated by HP precipitation and gel adsorption. As methods for quantitating individual C components in human blood are readily available, our initial studies are directed to defining the C profiles of ALA from human plasma. Levels of hemolytic complement components were found to decrease in heparinized plasma following gel adsorption or heparin precipitation, and no significant ALA remained in the supernatant fluid. In collaboration with Dr. H. J. Müller-Eberhard (Scripps Research Institute, La Jolla, Calif.), we are investigating the complement components in the gel eluate of heparinized human plasma, and the results will be published elsewhere. Thus far, C3, C6, factor B, properdin, IgG, and IgM, but not C5, have been detected by immunochemical methods. However, by hemolytic assay, C5 was present at approximately 10% of the heparinized plasma level, a value too low to account for the observed ALA. In view of the 35-fold reduction of protein in HPE_1, these data suggest selective concentration of complement components by gel adsorption.

Discussion. A number of mechanisms have been proposed to explain how cancer cells escape immunological inhibition. These range from simple outpacing of immune response to the shedding of cell surface antigens. Much emphasis has been placed on deficits of specific immune reactivity, both cellular and humoral, in accounting for progressive tumor growth. Our analysis of AKR leukemia and the antileukemia activity of normal plasma has led us to consider that complement, rather than specific antibody, may be a crucial limiting factor in the immune destruction of tumor cells (1). Several studies have in fact shown that complement levels are frequently depressed in animals and humans with cancer (11, 12). For this reason, provision of an exogenous source of complement, especially when blood levels are low, may be essential to successful cancer immunotherapy. Testing of this approach is constrained by the unphysiological protein burden imposed during the course of supplying adequate levels of complement through repeated plasma infusion. The stabilization, concentration, and purification of the ALA by heparinized plasma precipitation or gel adsorption is one step toward solving this problem.

Summary. Selective destruction of leukemic cells has been observed in AKR mice following infusion of normal serum, plasma, or whole blood. Heparinized plasma has significantly greater antileukemia activity than serum, and the active factor can be concentrated and purified by heparin precipitation or adsorption onto a calcium phosphate gel.

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