An Abnormal Hemolytic System Associated with Leukemia and Other Disseminated Malignant Diseases

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DURING the past ten years the phenomenon of "autohemolysis" has been studied in several laboratories[1-4] and a few investigators have developed it as a method to study the pathogenesis of hemolytic disease.[5-8] The technic is fundamentally simple. Blood is incubated for a time and then the hemolysis which occurred during incubation is measured. When normal blood is incubated for 24 hours at 37 C., few of the red cells are destroyed. But when spherocytosis is present a great deal of hemolysis occurs, especially if oxalate is used as the anticoagulant. In nocturnal hemoglobinuria oxalate prevents the hemolysis which otherwise occurs in PNH blood with no anticoagulant. The purpose of this report is to describe still another hemolytic system, one that is found in the blood of some patients with leukemia, lymphomata and other disseminated neoplastic diseases.

Materials and Methods

The basic procedure consisted of incubating one ml. of blood under mineral oil at 37 C. for 24 hours in a test tube 11 × 75 mm. After incubation the sedimented red cells were resuspended by gently rolling the tube between the palms to mix all free hemoglobin into the plasma. The tubes were then centrifuged and the plasma withdrawn. The plasma hemoglobin was measured by a quantitative benzidine reaction.[9] The blood was handled aseptically until incubation was completed. Modifications of the blood were carried out by standard procedures. Defibrination was accomplished by swirling the blood in an Erlenmeyer flask with a few glass beads. Aeration was accomplished by the same swirling. When it was desired to modify the plasma or serum without damaging the red cells (heating, deionization, addition of acid, etc.), the blood was centrifuged and the supernatant plasma was withdrawn for manipulation. The red cells were then washed with saline before the plasma and cells were recombined and incubated. Deionization was performed by passing the serum through an autoclaved column of cation exchange resin (IRC 50) on the sodium cycle or an anion resin (IRA 300) on the chloride cycle. The column contained 5 ml. of resin and was 20 cm. long. Analytic control indicated that virtually 100 per cent of Ca and Mg were removed. When normal and abnormal bloods were mixed, care was taken that there was no incompatibility. Additives were usually dissolved in saline. The concentration of these solutions was such that the addition of ten per cent by volume to the incubating blood gave the concentration desired in the plasma. Normal controls were used at all stages of the investigation. Many of the tests were done in duplicate and this has demonstrated that the results are reproducible within limits of ± 20 per cent. In interpreting the experimental data we have not considered as significant any change unless it exceeded these limits.

The blood for these experiments came from 15 patients with acute leukemia, 6 patients with Hodgkin's disease or reticulum-cell sarcoma, and one patient with multiple myelomatosis. The clinical aspects of the hemolytic system will be the subject of another report.

Calcium determinations were done by flame photometry.

Results

The incubation of normal defibrinated blood results in an increase of plasma hemoglobin by 5 to 10 mg. per 100 ml. Where this "neoplastic" hemolytic system

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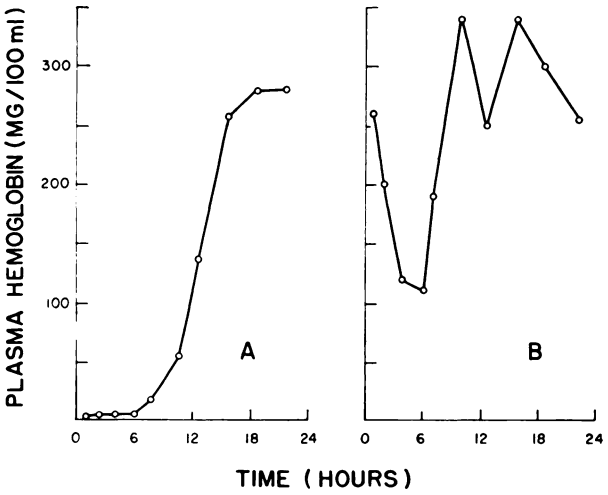

FIG. 1.—Hemolysis in vitro of defibrinated blood from a patient with disseminated reticulum-cell sarcoma. Twenty identical tubes were incubated at 37 C. **A.** At intervals one tube was withdrawn and the plasma hemoglobin was measured. There was little hemolysis for the first six or eight hours. **B.** At intervals one tube was removed long enough to resuspend the red cells; it was then replaced and incubation continued until the end of 24 hours at which time the plasma hemoglobin was measured. For the first six or eight hours resuspension affords some protection against hemolysis. Thereafter resuspension increases hemolysis over the amount that occurred in the undisturbed blood.

was found to be present the plasma hemoglobin was increased during incubation by 50 to 500 mg.

Differentiation. This hemolytic system was differentiated from other mechanisms of autohemolysis.

1. Complement-antibody hemolytic systems. The Coombs test in the subject hemolytic system is negative before, during and after incubation. The hemolytic system was not inactivated by preliminary heating of the serum at 60 C. for 30 minutes.

2. Spherocytosis, either hereditary or acquired, is characterized by intense hemolysis when blood is incubated with oxalate as an anticoagulant. Oxalate completely inhibits the subject hemolytic system.

3. Paroxysmal nocturnal hemoglobinuria (PNH) hemolytic system requires properdin and Mg^{++}. Serum treated with zymosan to remove properdin lost little or none of its hemolytic activity in the subject hemolytic system although it was completely inactive against PNH red cells. The same was true of serum treated with $BaSO_4$. Deionized serum was hemolytic if the calcium was replaced, but magnesium was not essential.

Characterization of the Hemolytic System

1. *Time of incubation* (fig. 1). After the blood is placed at 37 C. there is a latent period of 6 to 8 hours. Then hemolysis commences and continues rather intensely for 10 to 12 hours before it begins to subside. It is found that resuspending the red cells once during the early hours of the incubation affords them some protection against hemolysis. But after the latent period has elapsed resuspending the

TABLE 1.—*Cross-Incubation Experiment. The Patient Had Acute Monocytic Leukemia*

Plasma (1 ml.)	Red Cells (1 ml.)	Result (Mg. of Hb/100 ml.)
Patient	Patient	560
Patient	Normal	180
Normal	Patient	80
Normal	Normal	15

TABLE 2.—*The Effect of Leukocytes on Hemolysis*

Volume (ml.)	A		B	
	Plasma Hb (mg./100 ml.)	Leukocytes among the red cells (per cu. mm.)	Plasma Hb (mg./100 ml.)	Leukocytes among the red cells (per cu. mm.)
0.5	140	2,300	100	0
1.0	160	1,500	180	0
2.0	240	550	310	0

A. The heparinized blood was not manipulated except to place it in two identical sets of tubes. After 30 minutes the plasma and buffy coat were removed from one set and a leukocyte count was done on the sedimented red cells. The second set was incubated 24 hours at 37 C., mixed and centrifuged; plasma hemoglobin was measured.

B. Blood of the same patient one day later, lightly heparinized, stood until the red cells settled. The plasma was removed and centrifuged free of all cells. By repeated sedimentations in saline the red cells were freed of white cells. Packed red cells and plasma were then recombined and placed in two identical sets of tubes which were then treated as tubes A.

The intensity of hemolysis appears to increase in proportion to the amount of blood in each tube. The number of leukocytes trapped in the sedimenting red cells is less as the amount of blood increases. But the presence of leukocytes appears to have no effect on hemolysis, unless it be to inhibit it slightly.

Leukocyte count on the patient's whole blood was 3000 per cu. mm.

red cells does more harm than good, and even the addition of citrate at this time affords no protection.

2. *Site of the abnormality* appears to reside in the plasma or serum. When normal red cells are incubated in the serum of a patient, the cells are hemolyzed (table 1). The patient's red cells are also hemolyzed to some extent in normal serum as though they had been injured before the incubation.

3. *Leukocytes*, normal or leukemic, are not responsible for the hemolytic reaction (table 2). When a large amount of leukocytes was mixed with the blood before incubation they definitely inhibited hemolysis. In only one patient were the leukocytes destroyed during incubation. This man had an acute monocytic leukemia with 100,000 mature monocytes per cu. mm. of peripheral blood. The buffy coat disappeared during incubation of heparinized blood but it was preserved in oxalated or citrated blood. Hemolysis occurred only in the heparinized blood.

4. *Hematocrit* modifies the intensity of hemolysis (fig. 2). The greater the concentration of red cells the greater the proportion destroyed. In this case the intensity of hemolysis appears to be a function of the depth of the column of the sedimented red cells (table 2). This can be shown in other ways. When different volumes of the same blood are placed in identical test tubes there is more hemoly-

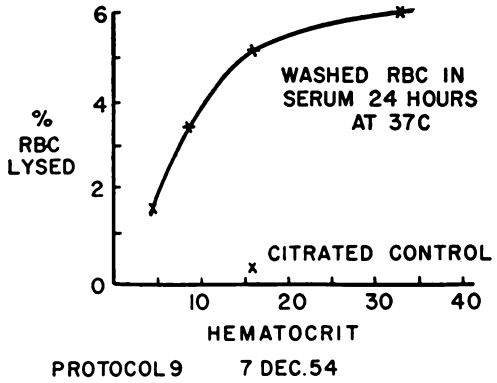

FIG. 2. The relation between hematocrit and the degree of hemolysis. Serum was added to defibrinated whole blood to achieve the several hematocrits. After incubation the hematocrit, total hemoglobin concentration and plasma hemoglobin concentration were determined. The proportion of red cells that had been hemolyzed was computed:

$$\frac{\text{Plasma Hb (mg.)} \times (100 - \text{Hct})}{\text{Hb (Gm. \%)} \times 1000}$$

sis with the greater volume. When the same volume of blood is placed in tubes of different diameter there is more hemolysis in the narrower tube.

5. *Coagulation* has no obvious effect upon the hemolytic system. When defibrination is carried out with aeration the hemolytic activity is no different from that of the same blood lightly heparinized to prevent clotting and similarly swirled as though to defibrinate. However, there is a difference between plasma and serum in this system. The hemolytic activity of serum and aerated plasma is resistant to heating but unaerated plasma is quickly inactivated (table 3). Anticoagulants which block Ca^{++} completely inhibit the hemolysis. Heparin has little or no effect except that in high concentrations it impairs but does not inactivate the hemolytic system.

6. *Aeration*, as indicated in paragraph 5 above, increases the intensity of hemolysis. This is not due to oxygen; it occurs when the blood is "aerated" with nitrogen. It is not due to loss of CO_2 *per se*. When the bicarbonate buffer system is stripped from the serum by adding HCl and applying vacuum until the pH is that of aerated serum without HCl (pH 7.8), there is little or no difference in the hemolytic activity of the two sera.

TABLE 3.—*The Effect of Preliminary Heating on the Hemolytic Activity of Plasma and Serum*

The plasma and serum were heated under oil at 60 C. for 30 minutes. Then they were cooled, the fibrinogen was removed from the plasma and washed leukemia red cells were added. The mixtures were incubated 24 hours at 37 C.

	Unheated	Heated
Aerated serum	310	500
Unaerated serum	100	160
Aerated plasma	410	440
Unaerated plasma	160	30

TABLE 4.—*Relation between Hemolysis, Glycolysis and pH*

Additive to whole blood	Test after incubation	
	pH	Plasma Hb (mg./100 ml.)
Saline	7.23	85
3-methylglucose	7.28	110
Glucose	6.63	10
Citrate	7.34	10

7. *pH* has an influence on hemolytic activity. When pH is raised, hemolysis increases until the pH exceeds 8.1. When the pH is lowered, hemolysis decreases until there is none at pH 6.8. The abrupt addition of too strong acid or base (e.g. 5% by volume of N/3 HCl or NaOH) causes a partial, irreversible loss of hemolytic activity.[7] Such materials should be added gradually with mixing.

8. *Glycolysis* decreases hemolytic activity. Increasing the plasma glucose to 500 mg. per 100 ml. completely inhibits hemolysis. Addition of the same amount of 3-methyl glucose (3-MG)—a nonmetabolizable analog—causes increased hemolysis (table 4). During incubation the pH of the glucose-rich blood falls, but not that of blood with 3-MG. When aerated blood is used the red cells of the 3-MG blood stay red and oxygenated; those of the glucose blood turn purple.

The results of incubation with various additives related to carbohydrate metabolism do not suggest that the hemolytic system is related to any fault of glycolysis or phosphorylation (table 5). The concentration of sodium azide and iodoacetic acid which increase hemolysis in the abnormal blood also cause hemoly-

TABLE 5.—*Effect of Additives in the Red-Cell Incubation Mixture*

Increases hemolysis	No effect on hemolysis	Decreases hemolysis	Blocks hemolysis
Iodoacetic acid* 2–4 mM/l	Iodoacetic acid* .2–1 mM/l	Sucrose‡ 700 mg./100 ml.	Glucose* 700 mg./100 ml.
Na azide† 10 mM/l	Na azide† 0.5–5 mM/l	Lactose‡ 700 mg./100 ml.	Fructose* 700 mg./100 ml.
3-methyl glucose* 700 mg./100 ml.	Sodium fluoracetate‡ 2 mM/l	Methylene blue* 0.1 mM/l	ATP‡ 15 mM/l
	2,4 dinitrophenol† 1 mM/l	ATP‡ 2–5 mM/l	
	Malonic acid‡ 2 mM/l	Sodium pyruvate‡ 25–100 mM/l	
	Inosine† 15 mM/l	Succinic acid‡ 2–10 mM/l	
	Sodium lactate‡ 15 mM/l	Malonic acid‡ 4 mM/l	
		Sodium lactate‡ 20 mM/l	

* Effects glycolysis.

† Effects phosphorylation.

‡ No effect on red cell carbohydrate metabolism. Most of these as additives remain outside the cell or represent the Krebs cycle which is absent from mature red cells.

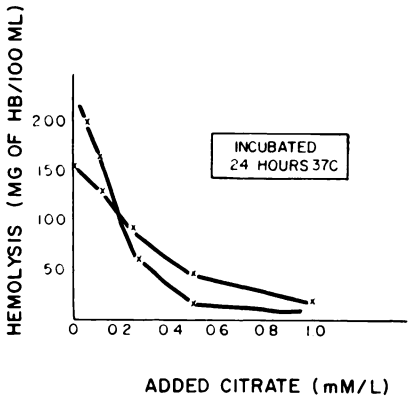

FIG. 3.—The effect of citrate to impede hemolysis. The curves represent two experiments; both patients had acute leukemia.

sis in normal controls. Even 3-MG permits some hemolysis in normal aerated blood. The concentration of ATP that is needed to block abnormal hemolysis is very great and is sufficient to complex the calcium.

Postprandial hyperglycemia is sufficient to inhibit hemolysis. Inhibition by normal serum[7] was traced to the glucose in that serum. In working with the hemolytic system it is necessary to obtain blood when the subjects are fasting.

Postprandial hyperlipemia has no effect on hemolytic activity.

9. *Calcium* appears to be essential to this hemolytic system. When Ca^{++} is removed by precipitation with oxalate, by chelation with citrate or by ion exchange, the hemolytic system is completely inactivated. It can be restored by addition of Ca^{++} (figs. 3 and 4). Ion exchange also removes Mg^{++} but its restoration is not essential. However, if Ca^{++} is restored to exactly the normal concentration the hemolytic activity remains a little diminished but is completely restored by Mg^{++}. An excess of Ca^{++} causes an excess of hemolysis. This can be

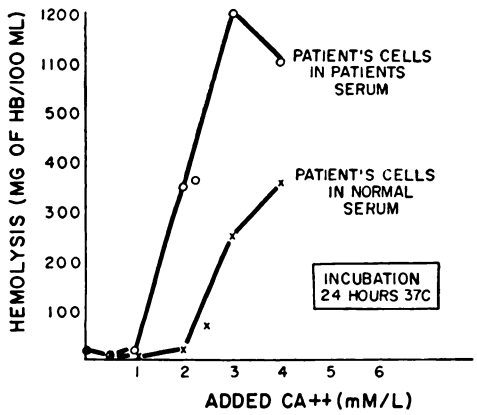

FIG. 4.—The effect on hemolysis of varying the concentration of calcium. The serum had been run through a cation exchange resin to remove all calcium. In a series of tubes varying amounts of calcium chloride were added. The two points not touched by the curve represent the original concentration of calcium in the two sera and the amount of hemolysis that occurred when the patient's red cells were incubated in those sera.

counteracted to some extent by glucose. Certain other cations, St^{++}, Zn^{++}, Ba^{++} and Co^{++} can be substituted for Ca^{++}.

Excepting one patient with myelomatosis whose serum calcium concentration was 14 mg. per 100 ml. none of these patients had a high serum calcium. Most of them had values at the lower limits of the normal range.

Normal red cells are hemolyzed when calcium is added to incubating blood. It is necessary to increase the concentration about 2 mM per liter above the normal to achieve this. The effect is enhanced by aeration and inhibited by glucose.

10. *Negative results.* There are no characteristic changes in morphology during incubation. In both normal and abnormal blood the cells first become crenated and then spherical. In both there occurs a small but consistent drop of the mean corpuscular hemoglobin concentration. Corrected for hemolysis, the MCHC decreases characteristically from a pre-incubation level of 35 per cent to 32 $\pm$ 1 per cent, indicating that the cells take up a little water. None of the abnormal bloods has been characterized by spherocytosis or other consistent aberrations of morphology. The plasma hemoglobin of unincubated blood has been below 3 mg. per 100 ml. in all of the patients. Preliminary treatment of abnormal serum with an anion exchange resin does not alter its hemolytic activity.

Discussion

This hemolytic system associated with leukemia and other disseminated neoplastic diseases apparently depends upon an abnormality of the plasma which results in hemolysis in the presence of calcium.

Hemolysis may be increased by: (1) preliminary aeration of the blood, (2) raising the pH, (3) depriving the system of glucose or substituting a nonmetabolizable glucose, (4) increasing the concentration of Ca, (5) preliminary heating of serum at 60 C. for 30 minutes, (6) resuspending the incubating red cells after 10 hours of incubation, (7) increasing the depth of the incubating red cell column.

Hemolysis may be reduced by: (1) providing an excess of glucose, (2) reducing pH, (3) reducing the concentration of Ca, (4) preliminary heating of unaerated plasma at 60 C. for 30 minutes, (5) resuspending the incubating red cells after 2 to 4 hours of incubation.

Change in pH appears to be a critical factor in most of the effects mentioned above. Aeration raises the pH and glycolysis lowers it. Glycolysis protects the red cells not only by lowering the pH but also by providing metabolic energy. When 3-MG is introduced it interferes with normal glycolysis and hemolysis is increased even though the pH of the 3-MG blood is the same as that of the control (table 3). The provision of ATP and inosine does not afford any considerable protection for the incubating red cells. ATP provides protection only when its concentration is great enough to suggest a binding of Ca^{++}. Interference with phosphorylation by such agents as sodium azide and iodoacetate does not render the red cells more susceptible to this abnormal hemolytic system. The cells are injured only by concentrations which also injure normal cells. This supports the concept that the abnormal hemolytic system involves a fault of the plasma rather than the red cells. However, the red cells are better able to resist hemolysis when provided with adequate glucose.

The state of Ca^{++} is an important factor related to the activity of the hemolytic system. The amount of hemolysis increases as the concentration of Ca^{++}. The reciprocal experiments in figures 3 and 4 demonstrate several interesting points: (1) In both figures the slope of the curves is steep, indicating that small differences in Ca concentration cause great changes in hemolytic activity. (2) There is no hemolysis at all until the concentration of calcium exceeds 1 mM per liter. (This is the concentration of Ca^{++} inside the red cell, a coincidence which may be without significance.) (3) When normal serum is used there is little hemolysis until an excess amount of calcium has been added.

The hemolytic system evidently involves a red cell-calcium reaction. The curves in figures 3 and 4 indicate that dissociated rather than protein-bound plasma calcium is involved. It is a nonspecific reaction since other divalent metals could be substituted for Ca^{++} and evoke a hemolytic reaction with the same characteristics: latent period and inhibition by glucose or citrate.

The nature of the plasma abnormality, its modification by preliminary heating, and its role in the hemolytic system are unknown. The fact that a hemolytic reaction with the above characteristics can be evoked in normal blood suggests that the "neoplastic" hemolytic system may be the result of a perversion or intensification of a normal response to incubation.

Summary

1. In the blood of some people with leukemia and other disseminated neoplastic diseases it is possible to demonstrate an abnormal hemolytic mechanism by incubating sterile blood in vitro at 37 C. for 24 hours. There is a latent period of 6 to 8 hours before hemolysis begins. Hemolysis can be inhibited by an excess of glucose or by low pH and also by removal of calcium. The heat stability of the system indicates that the plasmal abnormality is not enzymatic.

2. It is suggested that hemolysis is due to a reaction between ionic calcium and some component of the erythrocyte. The nature of the plasma abnormality that permits the reaction is not known.

3. The significance of this phenomenon in the pathogenesis of anemia in neoplastic disease has not been established.

Summario in Interlingua

1. In le sanguine de certe patientes con leucemia e altere morbos neoplastic disseminate, il es possibile demonstrar un anormal mechanismo hemolytic per incubar sanguine sterile in vitro a 37 C. durante 24 horas. Un periodo latente de 6 a 8 horas precede le comenciamento del hemolyse. Isto pote esser inhibite per un excesso de glucosa o per un basse pH e etiam per le elimination de calcium. Le thermostabilitate del systema indica que le anormalitate plasmatic in question non es relationate a enzymas.

2. Es formulate le these que le hemolyse resulta de un reaction inter calcium ionic e alicun componente del erythrocytos. Le nature del anormalitate plasmatic que permitte le reaction non es cognoscite.

3. Le signification de iste phenomeno in le pathogenese de anemia in morbo neoplastic ha non ancora essite establite.

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