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PRODUCTION OF HIGH-POTENCY CONCENTRATES OF ANTIHEMOPHILIC GLOBULIN IN A CLOSED-BAG SYSTEM*

Assay in Vitro and in Vivo

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REPLACEMENT therapy of classic hemophilia in this country is still handicapped by the low potency and high price of therapeutic materials. In this disease severely affected patients have less than 1 per cent of the mean normal circulating level of antihemophilic globulin (AHG, factor VIII). To stop various types of hemorrhage, minimum levels of 10 to 35 per cent must be achieved and maintained for one to twelve days. When one considers that frozen plasma has on the average 65 per cent of the antihemophilic globulin in the mean fresh sample, and when one further realizes that the half-life of transfused antihemophilic globulin in vivo is eight to eleven hours, it can readily be calculated that the volumes of plasma needed to achieve these goals often pose problems of circulatory overload.

Considerable progress has been made in England with large-scale production of concentrates of antihemophilic globulin from animal-blood sources. The antigenicity of these materials, which prevents their use beyond one short treatment period in any given case, has discouraged American workers from this line of attack. In contrast, considerable effort has been expended in this country to adapt the Swedish small-scale, sterile fractionation process to our large-batch programs.[1,2] The current result of this work is a commercial product that, on a protein basis, is about seven times as potent as whole plasma, and costs the patient $43.75 per 250 units of antihemophilic globulin (1 unit equals the activity in 1 ml. of normal fresh plasma).

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This report describes an entirely new approach to the production of a concentrate of antihemophilic globulin based on a report from this laboratory in 1959 that when frozen plasma is thawed in the cold, the cold-insoluble precipitate contains considerable quantities of the plasma antihemophilic globulin.[3] A more recent publication described exploitation of this fact for a large-scale pharmaceutical production process.[4] The difficulties encountered with that process, which led to the further work culminating in the new procedure described in this report, were as follows: a yield of only 25 per cent of the activity in the starting frozen plasma; and occasional batches that could not be sterile filtered.

This report will show that, utilizing the standard double plastic bag set, any blood bank or hospital can produce concentrates of antihemophilic globulin with a mean of thirty times the potency, on a protein basis, of frozen plasma, and in such a manner that the units of whole blood from which the concentrates were prepared can be "reconstituted" or not, as desired. The final product is potent, type specific and inexpensive. In addition to describing the production process, this report will analyze the first 41 infusions made into 4 hemophilic patients.

MATERIAL AND METHODS

Preparation of Cryoprecipitates Rich in Antihemophilic Globulin

Blood is collected from a donor into the ACD-containing bag of the standard 2-bag set. (The type of bag without heavy inner protection tubes has advantages in the later steps of this process.) The usual precautions concerning continuous mixing with the anticoagulant are especially important for conservation of antihemophilic globulin. The bags are spun in a cold centrifuge, and the supernatant plasma expressed into the satellite bag in the

customary way. A temporary clamp is placed on the tubing between the 2 bags, and the small bag is dropped into a mixture of dry ice and 95 per cent ethanol for fast freezing of the plasma; this will require ten to fifteen minutes. The 2 attached bags are then placed in the refrigerator for cold thawing of the frozen plasma. Approximately twenty-four hours later this will be complete, and the bags are returned to the centrifuge, cooled to 4°C. for sedimentation of the cold-insoluble precipitate at about 2000 g (maximum speed) for fifteen minutes. Since the precipitate clings to the bottom of the plasma bag, that bag may simply be inverted, and the temporary clamp removed, to permit the supernatant plasma to return to the red-cell bag from which it came. Both bags are then sealed and separated so that the "whole-blood" bag may be banked as usual while the flat, precipitate-containing bag is placed in frozen storage, where a number are stockpiled for future use. If the blood bank prefers to have 3 end products instead — precipitate rich in antihemophilic globulin, packed red cells and plasma poor in the globulin — this is accomplished by substituting a 3-bag set for the 2-bag set. (In this case one may accomplish freezing by laying the plasma bags flat on the solid-metal, "fast-freeze" shelf of a good freezer, instead of using a dry-ice mixture.)

At the time of administration, the number of bags to be used is removed from the freezer. Approximately 10 ml. of sterile, room-temperature, citrated saline solution (1 part of 3.8 per cent or 4 per cent sodium citrate to 5 parts of physiologic saline solution)* is added to each bag, making a total volume of about 13 ml. since the precipitate is typically about 3 ml. in volume. Gentle kneading and shaking dissolves the precipitates in five to ten minutes, after which all the solutions are pooled into 1 of the precipitate bags or into a transfer bag. Because of the potency of the material it is desirable to empty the bags completely; it is useful to roll them on a long test tube for the transfer process, but unless the bag is the type that can be flattened completely, some solution will remain behind. The typical solution prepared in this way has about 4 per cent protein concentration and good stability of antihemophilic globulin at room temperature.

Assay in Plasma

Assay for antihemophilic globulin was carried out by the method of Pool and Robinson.[5,6] The standard for activity was the tentative pooled-plasma standard supplied by the Division of Biologics Standards, National Institutes of Health. Comparison of this standard with 30 normal donors in our laboratory gave it a value of 102 per cent of the mean freshly collected normal plasma.

*Available commercially from Cutter Laboratories, Incorporated, Berkeley, California; sterile, pyrogen-free 4 per cent sodium citrate (Don Baxter, Incorporated, Glendale, California) may also be used to prepare citrated saline solution.

Assay in Concentrates

As recently reported from several laboratories, assay methods that work well for whole plasma may give misleading results when applied to concentrated sources of antihemophilic globulin.[6-8] The closest estimate of the in vivo potency of this concentrate was obtained when it was prediluted 1 part to 9 parts of hemophilic plasma before being substituted in the standard plasma assay, but still the values were consistently too low. To predict in vivo potency, as pointed out below, a correction factor of 1.4 must be applied to the in vitro assay value. In contrast, in assay of the pharmaceutical preparation that we previously described, predilution in hemophilic plasma resulted in an overestimate of its potency by a factor of 2; in that case the most useful procedure was predilution in aged citrated human serum. Thus, it appears that, at the present time, it is necessary to determine activity of a given type of concentrate by in vivo studies; then an appropriate in vitro procedure can be chosen for subsequent studies.

Assay of Other Clotting Factors in the Cryoglobulin Precipitate

Fibrinogen was assayed by the procedure of Jacobsson,[9] true prothrombin and factor VII by the methods of Owren and Aas,[10] factor V by that of Borchgrevink et al.,[11] factor X by that of Hougie,[12] and factors IX, XI and XII by the partial-thromboplastin-time methods of Rapaport and his coworkers.[13]

Patient Studies

The circulating response to antihemophilic globulin of hemophilic patients admitted to the hospital for treatment of hemorrhage was followed. Typically, a Deseret Intracath was put in place and kept patent with a continuous drip of 5 per cent dextrose or physiologic saline solution. With the 2-syringe technic samples were taken from the catheter before, and five minutes after, each infusion into acid-citrate anticoagulant (9 parts of blood to 1 part of the anticoagulant, which was made up by combination of 2 parts of 0.1-M citric acid with 3 parts of 0.1-M sodium citrate).

After the transfer bag was loaded with the cryoglobulin solutions, it was shaken gently to mix, and a small sample was expressed for assay. The input tube was then tied off and cut, and the bag and its contents weighed. At the end of the infusion the empty bag was also weighed to determine the volume administered, by difference.

Results

Preliminary studies, already reported, were carried out on outdated blood to gather data on the

feasibility of the process, on the degree of concentration of antihemophilic globulin and on the effect of variables in handling on that concentration.[6,14] During these studies, when there were no in vivo data for comparison, the samples of concentrate were handled for assay as if they were ordinary plasma samples. As a result, a mean of fifteen-and-one-tenth times concentration of antihemophilic globulin was observed when the concentrate was compared with a duplicate of the whole thawed plasma from which it was prepared, at the same protein concentration. Only about 26 per cent of the concentrate protein was clottable with thrombin.

On the basis of these preliminary results, production of the concentrates from freshly collected blood was instituted at the Peninsula Blood Bank, Burlingame, California. Cryoprecipitates from donors of Group A or O blood were stocked and then used in patients who required treatment. Table 1 summarizes the data obtained from 41 infusions in 4 patients. When it was found that the in vitro assay consistently underestimated the potency of each preparation as back calculated from the rise in circulating level of antihemophilic globulin and the patient's plasma volume several different preparative technics for handling the concentrate in in vitro assay were tried. Whether the concentrate was prediluted simply in citrated saline solution (as for plasma samples), in citrated aged human serum (as for the pharmaceutical concentrate) or in hemophilic plasma (as recommended by Biggs), the assay always underestimated the in vivo response, but the last procedure came closest to the in vivo value. In these studies it was decided not to enter the closed-bag system to get a sample of the plasma. A potency comparison with thawed, fresh-frozen plasma must therefore be based on our own data and those recently reported by Biggs et al.,[15] showing that such plasma typically has about 65 per cent of the activity of antihemophilic globulin present in fresh plasma. Therefore, the mean concentration value of nineteen-and-eight-tenths times fresh plasma becomes thirty-and-four-tenths times typical frozen plasma.

Figure 1 shows the levels of antihemophilic globulin in a patient (M.W.) who entered the hospital with a retroperitoneal hemorrhage and was infused

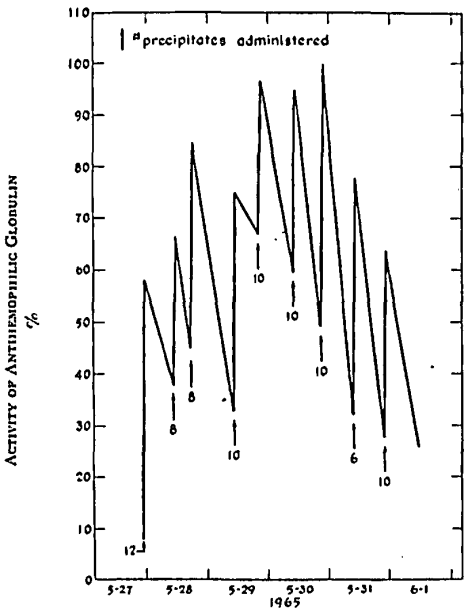

FIGURE 1. *Response of Circulating Antihemophilic Globulin in a Patient (M.W.) during Cryoprecipitate Infusions (Patient's Plasma Volume, 3000 Ml., as Calculated from the Technic of Cr^{51}-Labeled Red Blood Cells and the Hematocrit).*

every twelve hours with the number of precipitates indicated. The volume of each infusion can be easily estimated if the number of precipitates is multiplied by 13 ml. From these and similar data in other patients the mean biologic half-life was found to be thirteen-and-one-tenth hours, with the variability shown in Table 1.

Table 2 presents the determination of other clotting-factor concentrations made in 2 particular pools selected at random. It appears that there is no significant concentration of any clotting factor other than antihemophilic globulin and fibrinogen.

Similarly, immunoelectrophoresis patterns of several pools were identical to those of whole plasma and did not indicate total deletion of any plasma protein component.

In Table 3 are shown the current costs to patients

TABLE 1. *Assays in Vitro and in Vivo on 41 Cryoprecipitate Infusions (Pools of 3 to 12 Solutions).*

Measurement of Variability	Activity of Antihemophilic Globulin in Vitro (34 Infusions)	Activity of Antihemophilic Globulin in Vivo (41 Infusions)*	Protein Concentration of Infused Cryoprecipitate Solutions (36 Infusions)	Activity of Antihemophilic Globulin X Fresh Plasma from in Vivo Assay (41 Infusions)†	Half-Time in Vivo	Clottable Protein in Cryoprecipitate Solutions (10 Infusions)
	%	%	*mg./ml.*		*hr.*	%
Mean	843	1211	42.0	19.76	13.1	33.3
S.D.	221	549	4.95	8.89	4.84	5.7
Range	370-1428	657-2830	32.8-58.6	8.77-50.9	8.0-24.0	24.5-39.8

*Back calculated from rise in antihemophilic globulin & patient's plasma volume.
†Protein basis.

TABLE 2. *Assays of Other Coagulation Factors in 2 Randomly Selected Cryoprecipitate Solutions.*

FACTOR	SAMPLE A	SAMPLE B
	%	%
I (fibrinogen)	—*	—*
II (prothrombin)	27	35
V (proaccelerin)	<1	<1
VII (proconvertin)	33	32
IX (PTC)	100	100
X (Stuart-Prower)	25	29
XI (PTA)	20	72
XII (Hageman)	37	33

*See Table 1.

of the 4 types of concentrated antihemophilic globulin available in this hospital. Since no whole blood is consumed in the production of the cryoprecipitates the blood bank accepts blood in payment at a ratio of 1 unit of whole blood to 4 cryoprecipitates and does not impose a service charge. Another major consideration, of course, is the yield of antihemophilic globulin in the final concentrate as a percentage of what was present in the original plasma. For fresh-frozen plasma this is 65 per cent on the average; for the commercial pooled lyophilized plasma it is presumably the same, the high final levels being the result of screening and selection of high-level donors. Although the small-scale (about 5 liters per batch), sterile process in Swedish hands gives the remarkably high yield of 87 per cent of fresh plasma activity[16] the yield for the large-scale version of the same process in this country is typically less than 25 per cent of that in the starting frozen plasma, or 16 per cent of that in the original fresh plasma. The cryoprecipitate process provides a yield of 70 per cent of the antihemophilic globulin present in the frozen plasma from which it was made, or 46 per cent of that in the fresh plasma.

A large pool of outdated plasma was subdivided into 250-ml. aliquots, and a cryoprecipitate was prepared and frozen from each. Periodically, one is taken from the freezer, thawed and assayed. At the time of this writing six months of storage have resulted in no measurable decay of activity of antihemophilic globulin.

TABLE 3. *Comparison of Costs to Patients of Currently Available Sources of Antihemophilic Globulin.*

SOURCE	ACTIVITY OF ANTIHEMOPHILIC GLOBULIN X PLASMA (AT EQUIVALENT PROTEIN CONCENTRATIONS)	COST FOR 250 UNITS OF ANTIHEMOPHILIC GLOBULIN†	
	%	$	*ml. of donated blood*
Fresh-frozen plasma*	0.65	19.30	770
Pooled lyophilized* commercial "antihemophilic plasma"	1.0	1.00	500
Commercial "AHG-rich fibrinogen"	6.8	43.75	—
Cryoprecipitates*	19.8	—	272

*Rates established at Peninsula Memorial Blood Bank, Burlingame, California.

†1 unit = amount in 1 ml. of average fresh normal plasma.

DISCUSSION

Although at first glance a single-unit process for production of these concentrates seems far less efficient than a large-bulk process it is possible that this need not be so. The local blood bank, with 2 4-place centrifuges available, was able to add production of 64 concentrates per week to 1 technologist's regular schedule. And since the yields are much higher than those of large-scale processes, which require sterile filtration as a final step, a large number of blood banks can presumably easily convert as much (or more) antihemophilic globulin to the concentrated form as a few pharmaceutical houses could.

We do not recommend maintaining the average patient at the high levels depicted in Figure 1. Those infusions were made over a weekend during which assays were not performed; the patient's plasma samples were frozen in small aliquots and assayed during the following week, when we discovered how high his circulating levels had become. Because of the logarithmic decay the amount of antihemophilic globulin destroyed per unit of time will be proportional to the circulating level; thus, it would be considerably more economical to maintain a patient between 30 per cent and 60 per cent on a twelve-hour-infusion basis than to keep him between 50 per cent and 100 per cent. If more frequent infusions are scheduled the "floor level" can be maintained still more economically. In the recent report cited above Biggs et al.[15] recommended a floor level of 25 per cent to be maintained for five days after extraction of several teeth in a hemophilic patient, and this may be a reasonable goal for most hemorrhagic challenges.

The biologic half-life of this activity in the circulation of these patients was a bit longer than expected, with a very large range of eight to twenty-four hours. We can only speculate at present that the variability results from the amount of bleeding going on in the patient, from some characteristic of the patient's clotting-factor turnover pattern or from the amount of denaturation of the protein that may have occurred during preparation of the concentrate. It is even possible that antihemophilic globulin prepared by this very simple process and stored in the frozen state at a very high protein concentration may have better resistance to denaturation than that of fresh-frozen plasma, our previous standard for determination of turnover rate of antihemophilic globulin in hemophilic recipients.

Some comment should be made on the fairly large standard deviations observed in the data presented in Table 1. This seems a necessary accompaniment to single-unit processing; circulating levels

of antihemophilic globulin show a remarkably large variability in the human population (50 to 200 per cent of the mean), and this fact, combined with possible processing variables, means that cryoprecipitates may be very different from one another. The chance of preparing a very high or a very low pool of antihemophilic globulin decreases, of course, as the number of precipitates in a particular pool increases.

It is disappointing that no simple modification of our standard assay procedure gives a good estimate in vitro of the in vivo potency of these preparations. Although assay of antihemophilic globulin in plasma has reached a high general level of accuracy, that of concentrates still poses many problems, and each new concentrate appears to have unique characteristics. These may depend on what other proteins happen to be in the fraction, and at what concentrations, but at present no a priori considerations permit us to select an appropriate procedure. With our determinations of in vivo potency we could arrange departures from our normal process that result in higher readings; one might simply be, for example, carrying out the aluminum hydroxide adsorption step on undiluted instead of on 20 per cent plasma, since we know that this markedly increases assay values. At present it seems adequate, however, to run the assay in the usual way and to apply the correction factor determined from the whole series of in vitro, in vivo comparisons.

This preparation has no value for therapy of any coagulation-deficiency disease other than classic hemophilia since there is no evidence of concentration of any additional factor with the exception of fibrinogen. Commercial fibrinogen preparations that are 90 per cent clottable are available, so that the present one has no advantage for that purpose.

No systematic review of the observed clinical responses will be presented here, but, in summary, no side reaction of any kind was observed and clinical improvement was entirely commensurate with the observed rises in circulating levels of antihemophilic globulin.

Studies just completed by L. N. Button, of the Peter Bent Brigham Hospital, Boston, show the in vivo survival of labeled red cells autotransfused after 21 days' storage in 3 units of reconstituted blood prepared by this process averaged 86.3 per cent, with a range of 83 to 88 per cent. These values are well within the normal range.

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EFFECT OF AMINOPHYLLINE ON RESPIRATORY-CENTER SENSITIVITY IN CHEYNE–STOKES RESPIRATION AND IN PULMONARY EMPHYSEMA*

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ALTHOUGH aminophylline has been known for many years to abolish Cheyne–Stokes respiration and restore a normal breathing pattern, the mechanism of this phenomenon is poorly understood. This agent acts either by stimulating the respiratory center directly or by increasing its sensitivity to carbon dioxide.[1,2] Recent studies of aminophylline in patients with Cheyne–Stokes respiration associated with congestive heart failure have shown that the agent reduces blood flow and oxygen consumption of the brain.[3] The elimination of periodic breathing in these subjects was thought to be due to the decrease

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