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REVIEW

Specific Hemoperfusion Through Agarose Acrobeads

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ABSTRACT

Agarose acrobeads were produced by encapsulating polyacrolein microspheres (acrobeads) of 0.2 μm average diameter within an agarose matrix. Crosslinked agarose acrobeads of diameters ranging from 0.5 to 0.8 mm were found to be optimal spheres for specific hemoperfusion purposes. Agarose provides the biocompatibility and mechanical strength of the agarose acrobeads. Acrobeads contain a high aldehyde-group content through which various amino ligands, i.e., proteins, antigens, antibodies, enzymes, and so on, can be covalently bound in a single step under physiological pH (or other pH). Thus, antibodies, antigens, or toxic materials may be directly removed from whole blood by hemoperfusion. During in vitro and in vivo hemoperfusion trials, the content of erythrocytes, leukocytes, and thrombocytes was essentially unaltered. Likewise, a battery of the soluble blood components (Cl^-, K^+, Na^+, Ca^{2+}, PO_4^{3-}), total proteins, albumin, and C_4' component of the complement cascade, as well as the enzymes SGOT, LDH, and alkaline phosphatase, remained constant within narrow limits during the hemoperfusion procedure. The chemical and physical structure of the beads is stable; neither acrolein nor bead fragments were detected in hemoperfusion trials. Similarly, leakage of antibody bound to the agarose acrobeads into the blood is insignificant.

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Thus far, we have demonstrated the efficacy of the crosslinked agarose acrobeads for extracorporeal removal of "unwanted" substances from whole blood in the following systems: (a) removal of specific antigens (digoxin or paraquat removal with antidigoxin or antiparaquat antibodies bound to the acrobeads, respectively), (b) removal of specific antibody (antiBSA) removal with BSA bound to the beads), (c) removal of immune complexes (BSA–antiBSA complex removal with C1q bound to acrobeads), and (d) removal of specific metals (removal of iron with deferoxamine bound to the agarose acrobeads).

Index Entries: Agarose–polyacrolein microsphere beads; agarose acrobeads; polyacrolein microspheres; hemoperfusion; hemoperfusion with agarose–polyacrolein microsphere beads; hemoperfusion with agarose acrobeads.

INTRODUCTION

The extracorporeal techniques: hemodialysis, hemoperfusion with charcoal or ion exchange resins, transfusion, and pharesis (of plasma erythrocytes, leukocytes, or thrombocytes) are routine procedures in clinical medicine. The procedures replace essential blood components or remove toxic materials from the blood. However, hemoperfusion appears to be the simplest, most direct method for detoxification of blood. The large number of patients on long term hemodialysis and the increasing numbers of patients requiring specific blood-soluble components or blood-formed elements spurred commercial development of simpler to use, more efficacious, and cost-effective equipment. Thus, state-of-the-art extracorporeal instrumentation already available and in current use in hematology and nephrology departments and emergency rooms may be used for hemoperfusion. The next breakthrough in the medical use of hemoperfusion lies in the field of adsorbents.

Activated charcoal (*1*) and nonionic resins (*2,3*) are the principal sorbents commercially available to remove toxic molecules directly from the blood stream. Both are biocompatible following encapsulation in albumin collodion (*4*), nylon (*5*), hydroxyethyl methacrylate (*6*), or agarose (*7*). Thus far, these adsorbents have been employed mainly for medical emergencies: hepatic failure (*8*), renal failure (*9*), drug self-abuse (*10,11*), iatrogenic overdose (*11*), and metal poisoning (*12*). Dozens of other uses, limited only by the imagination of the investigator–clinician, have been tried or suggested, *see* reviews (*13–17*). Unfortunately, not only are charcoal and nonionic resins nonspecific, but they indiscriminately remove physiologically desirable biocompounds (*18*) in addition to toxic substances, and their affinity toward the toxic compounds is low in many instances.

Several experimental in vivo systems for elimination of specific antigens or antibodies have been described. In one, blood flows through a

tube coated with entrapped immunosorbent (*19*). In another (*20*), antigen is bound to derivitivized nylon tubing. The altered tubing itself is passed into the vascular bed to remove specific antibody during blood circulation. In a third, sorbent is coupled to nylon, encapsulated, and placed into a column through which blood is hemoperfused (*5,21*). And, of course, commercially packaged systems containing charcoal or nonionic resins are available.

A more recent, but very exciting approach, which seems to be clinically successful, is the use of (a) Protein A bound to charcoal collodion for therapy of solid tumors (*22*), and (b) a heat-killed, stabilized *Staphalococcus aureus* sorbent to remove serum IgG in an autoimmune hemolytic-anemia syndrome (*23*).

A major drawback of many of the specific systems is their disruptive effect on the formed elements of the blood. To circumvent this, solely plasma is perfused through the sorbents (plasmapheresis), requiring additional, sophisticated equipment to separate cells from plasma, subsequently reconstituting them. In other cases, additives, i.e., prostaglandins or citrate, have been infused to obviate this problem (*17,24*).

We have designed and produced a specific sorbent system, Agarose–Polyacrolein Microsphere Beads (agarose acrobeads). These are agarose beads of 500–800 μm in diameter (or of larger or smaller diameter, fabricated according to specific design), containing thousands of acrobeads of 0.2-μm mean diameter, encapsulated within an agarose matrix. These seem to be the optimal size range for hemoperfusion; although we can now produce agarose acrobeads of any given diameter ranging from 40 μm to 1 cm. A wide variety of proteinaceous and other molecules may be bound covalently to these acrobeads (*25–27*). For example, a specific antigen is covalently bound in a single step to the agarose acrobeads to remove a specific antibody or vice versa. The gel matrix/coating confers physical strength, biocompatibility, and spatial configuration, as well as porosity, to permit rapid entry of molecules for reaction. Thus, medically useful acrobeads with designated properties are available. So far, agarose polymercaptal microspheres for removal of mercury from blood and other fluids have been produced (*28,29*). Immunospheres with specific antibodies on their surface have been used for labeling and separating T from B lymphocytes (*30,31*). In addition, magnetic properties can be conferred on the acrobeads so that cells can be labeled and separated in a magnetic field (*32*). Furthermore, acrobeads have been used for affinity chromatography (*26*), cell culturing (*33*), and diagnostics (*34*).

To the best of our knowledge, our previous reports are among the very few, if not the first, to successfully remove specific, undesired compounds (antibodies, antigens, metallic cations, herbicides, and drugs) from whole blood in vivo by hemoperfusion through immobilized solid spheres. Our system for hemoperfusion is gentle and does not significantly affect the formed elements and soluble components of the

whole blood. Chang et al. (*16*) has performed clinical trials with semipermeable microcapsules. Although hemoperfusive trials to remove toxic compounds have been reported by other investigators, most of these were achieved with nonspecific adsorbents, such as activated charcoal, ion exchangers, or agarose that contained materials such as fuller's earth or ion exchangers. Many other trials employed plasma perfusion rather than hemoperfusion because the adsorbents possessed either poor flow properties or were too disruptive of the formed elements of the blood.

We have demonstrated the potential uses for the crosslinked agarose acrobeads for specific hemoperfusion in the following systems: (a) removal of specific antigen, i.e., digoxin removal with antidigoxin antibodies bound to the beads and paraquat removal with antiparaquat antibodies bound to the beads; (b) removal of specific antibody, i.e., antibovine serum albumin (BSA) antibody removal with BSA bound to the beads; (c) removal of specific immunocomplexes (IC), i.e., BSA–antiBSA, with C1q bound to the beads; and (d) removal of specific toxic cations, i.e., iron removal with deferoxamine bound to the acrobeads.

We review herein our animal hemoperfusion model systems, as well as our immunosorbent systems, which are capable of removing specific, "unwanted" materials from the blood stream, without significantly altering other blood components.

PREPARATION OF THE AGAROSE ACROBEADS–LIGAND

Preparation of the Agarose Acrobeads

Synthesis of the Acrobeads

The acrobeads are formed by the irradiation (cobalt source) of acrolein in the presence of an appropriate surfactant, i.e., polyethylene oxide (*35*).

Production of the Agarose Acrobeads

The acrobeads are matrix encapsulated within agarose (*25,26*).

Crosslinking the Agarose Acrobeads

The agarose acrobeads are cross-linked by reacting them with divinyl sulfone under alkaline conditions (*36*). Agarose acrobeads formed in this manner are stronger; more resistant to agglomeration and shattering and sterilizable by autoclaving.

Preparation of Agarose Acrobeads Bound with Amino Ligands

The agarose acrobeads are reacted with the appropriate amino ligand [anti-paraquat, antidigoxin, BSA, C1q, and deferoxamine (*25, 26,37–42*)]. Unbound ligand is removed by filtration-wash; remaining free aldehyde groups on the acrobeads are blocked with ethanolamine or

TABLE 1
Binding Capacity of the Agarose Acrobeads[a]

Ligands	Binding capacity, mg/g beads
BSA[b]	15
Rabbit antidigoxin	6
Deferoxamine[c]	80
C1q	5
Rabbit antiparaquat	18

[a]One mL agarose acrobeads in 5 mL saline (or PBS) was reacted for approximately 12 h at room temperature with an excess amount of the desired ligand. Unbound ligand was washed out by repeated decantation.
[b]BSA was bound through a spacer arm of polylysine–glutaraldehyde.
[c]Deferoxamine was bound at pH 8.5 at 60°C for 3 h.

serum albumin appropriate for the species to be hemoperfused. The agarose acrobeads are washed exhaustively to remove reactants and stored in merthiolate/saline or azide/saline (*25,26*).

Binding a Spacer Arm of Polylysine Glutaraldehyde:

Where spacer arms were required, acrobeads were reacted with polylysine and washed free of unbound polylysine, then reacted with glutaraldehyde and washed free of unbound glutaraldehyde.

Binding capacities of the above amino ligands to the agarose acrobeads are shown in Table 1.

HEMOPERFUSION PROCEDURE

A schematic diagram of the hemoperfusion system is shown in Fig. 1. The "software" (cannulas, tubing system, and column), as well as the hemoperfusion procedure, were previously described by us in detail for rabbits (*43*) and for dogs (*39*).

The hemoperfusion tubing and pumping system (*43,39*) used is as follows: The *in situ* cannulas are connected to the specially designed hemoperfusion arterial outlet set via a three-way stopcock. Arterial blood flowed from the appropriate artery through the pump segment sited in the blood pump to perfuse the beads in the column. The other side of the column was attached to the venous inflow set, consisting of a bubble trap, Tee connection, and tubing that ends in a three-way stopcock. Blood is returned to the animal via a stopcock to the cannula in the appropriate vein.

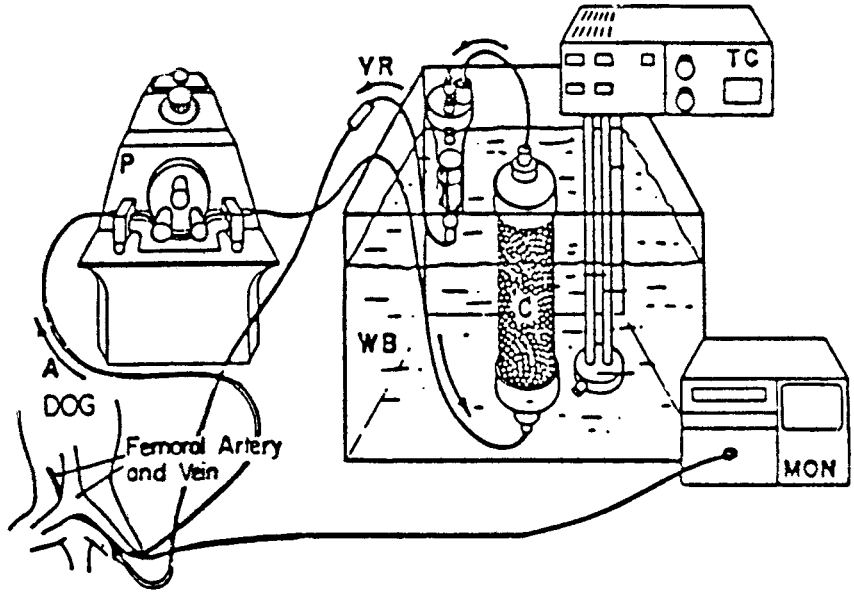

Fig. 1. Schematic diagram of hemoperfusion procedure in dogs; not drawn to scale. A = arterial flow, P = peristaltic pump, C = column, B = bubble trap, VR = venous return, TC = temperature control and water circulator, MON = blood pressure monitor, WB = water bath for constant blood temperature.

SAFETY STUDIES AND BIOCOMPATIBILITY

Stability of the Covalent Polyacrolein–Antibody Bond

Possible leakage of antibody from the beads was tested during three hemoperfusion trials in nonintoxicated dogs. In three separate studies we found insignificant levels of leached antibody (Table 2). A possible reason that antiparaquat antibodies were not detected is that a ^{14}C-label was used in this RIA in contrast to a ^{131}I-label for the antidigoxin RIA. Thus, antiparaquat antibodies might have been below the limit of detection. In any case, that level was extremely low.

If protein leaked from the beads to evoke an antigenic response, we might expect, at least indirectly, a decreased efficiency of digoxin removal in dogs participating in repeated hemoperfusion trials; i.e., if they produced antidigoxin antibodies that competed with the digoxin for sites on the agarose acrobeads. We have not seen any decrease in the efficiency of digoxin removal in dogs that participated in up to four hemoperfusions. Protein leakage may be a serious problem when cyanogen bromide coupling is employed for binding protein to the sorbent. It should be mentioned here that in the experimental studies leading to their proposal for the mechanism of cyanogen bromide activation, Kohn and Wilchek (*44*) developed newer cyano-transfer reagents to produce

TABLE 2
Leakage of Antibodies Bound to Agarose Acrobeads During Hemoperfusion in Dogs[a]

Exp No.	Antibody	Antibody/mL plasma, ng	Antibody/dog, μg	Antibody released, %
1	Antidigoxin	0.29	0.20	0.16×10^{-3}
2	Antidigoxin	0.20	0.17	0.14×10^{-3}
3	Antiparaquat	0.0	0.0	0.0

[a]Dogs were hemoperfused for 180 min at a blood flow rate of 120 mL/min. Column contained 25 g agarose acrobeads–antidigoxin or –antiparaquat. 60 mL whole blood samples were drawn at the beginning and end of the hemoperfusion trial. Antibody content of the plasma was quantified by RIA (^{131}I for antidigoxin; ^{14}C for antiparaquat).

activated resins free of imidocarbonytes and carbamates, but not to obviate the leakage problems.

More directly, we examined the blood of a dog that had participated in three hemoperfusions for anti-antidigoxin antibodies and for antirabbit IgG (the source of the antidigoxin) by Ouchterlony immunodiffusion before the second hemoperfusion (3 wk after the first hemoperfusion) and before the third hemoperfusion, (4 wk after the first hemoperfusion). The time elapsed was sufficient to evoke anti-antibodies if they were produced. No reaction was seen in the gel. Specific rabbit antidigoxin and rabbit IgG gave strong lines of precipitate. Thus, we do not anticipate an antigenic response in animals exposed to the antibodies on our column, under our conditions.

PHYSICAL STABILITY OF THE AGAROSE ACROBEADS

Normal saline was pumped through the agarose acrobeads in a typical in vitro hemoperfusion. The fluid was sampled periodically and assayed for turbidity by nephelometric methods to determine whether disrupted particles or microspheres were released. Limits of detection for microspheres was 25 ng/mL. No particles or acrobeads were detected (*42*). The fluid was also microassayed for sulfur to determine loss of the crosslinker, divinyl sulfone; none was detected (*42*). Release of acrolein from the agarose acrobeads into solution was examined in beads stored in saline at 4°C. Possible released acrolein was measured by UV absorbance at 210 nm of samples taken periodically over a period of 4 mo (*42*). The sensitivity of this method is 0.5 ppm. No acrolein was detected in the menstruum.

In our hands, the agarose acrobeads–detoxifying ligands are stable. For example, agarose acrobeads–BSA have been used in many hemoperfusion trials, were stripped of sorbed molecules, reused at least a dozen times, and still retained their capacity after 15 mo.

SPECIFICITY OF THE AGAROSE ACROBEADS–BSA SYSTEM

After perfusion of normal rabbit serum through the agarose acrobeads–BSA and rinsing, no protein was eluted from the beads, using our standard elution methods. Protein in the eluate was determined spectrophotometrically at 280 nm. Thus, it is shown that these sorbents do not adsorb proteins nonspecifically (*26,27*).

BIOCOMPATIBILITY OF THE AGAROSE ACROBEADS–LIGAND

The formed elements and a battery of soluble components of the blood were sampled during the course of most hemoperfusion trials. Figure 2 shows erythrocyte, leukocyte, and thrombocyte counts, as well as the hematocrits obtained during typical hemoperfusions of intoxicated dogs. A slight, transient decrease in leukocyte and platelet counts was noted, but their number stabilized and returned, more or less, to prehemoperfusion values before termination of the hemoperfusion. Bleeding diatheses were never observed. Similar results were obtained in all other clinical trials.

Soluble components of the blood were also sampled during most hemoperfusion trials. Figure 3 shows mean values and range of relevant soluble components of the blood during 10 hemoperfusion trials. There was little variation in the ionic species, including Ca^{2+}, Na^{+}, Cl^{-}, and PO_4^{3-}. K^{+} tends to rise from the low normal values to normal mean values. Hypocalcemia is not unusual during hemoperfusion with ex-

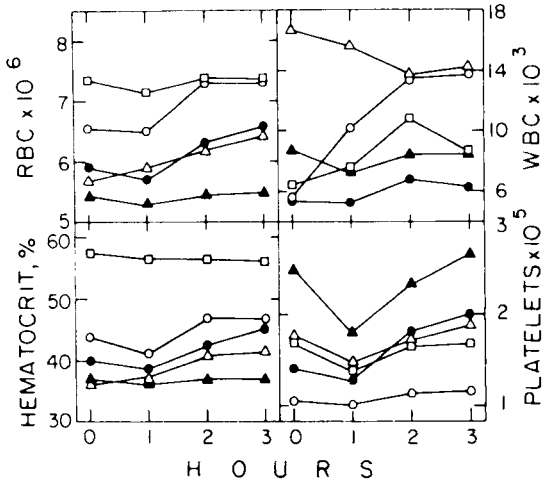

Fig. 2. Various parameters of the formed elements of the blood of five dogs during hemoperfusion trials. Dog wt = 15–25 kg. 25 g agarose acrobeads–antidigoxin/column. Blood flow rate = 120 mL/min.

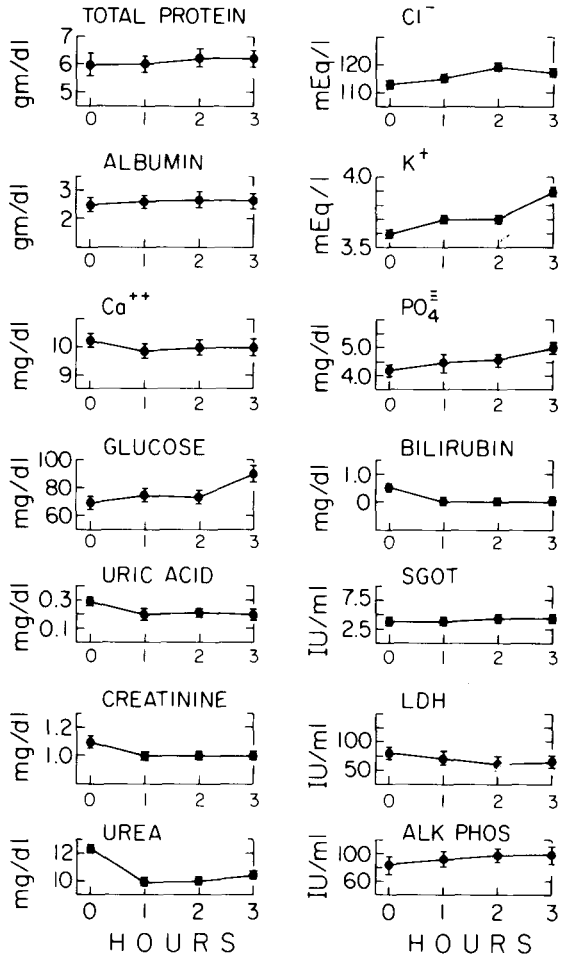

Fig. 3. Limits of variability of routine parameters of the soluble components of the blood during 10 hemoperfusion trials. Dog wt = 15–25 kg. 25 g agarose acrobeads–antidigoxin/column. Blood flow rate = 120 mL/min.

change resins or charcoal (*45*). None was seen here. Kidney functions, as expressed by creatinine, urea, or uric acid levels, remained normal. Similarly, liver function tests, serum enzymes, serum proteins, and pH remained within normal limits. Bilirubin, creatinine, uric acid, and urea fell somewhat. The largest deviations from the published "normal" range prior to the start of hemoperfusion were seen in albumin and total protein content; presumably a function of our dog population. Significant changes did not occur during hemoperfusion.

Complement

A significant system of the blood that must be quantified when proving biocompatibility is the complement system. C_4' was examined during seven different hemoperfusions; no significant depletion in C_4' was observed (Table 3).

TABLE 3
Complement (C_4') Content of the Plasma During Hemoperfusion in Dogs[a]

Expt #	1	2	3	4	5	6	7
Minutes	Complement, ng/mL						
0	52	52	48	25	48	44	25
30	61	56	49	23	46	42	31
60	66	52	50	31	40	32	32
120	48	52	56	42	60	36	25
180	52	58	56	36	52	56	24

[a]Dog wts, 20–25 kg; agarose acrobeads–antidigoxin, 25 g/column; blood flow rate, 120 mL/min. C_4' was assayed by Radial Immunodiffusion with either dog or human anticomplement.

Hemoperfusion Trials with Sterile Agarose Acrobeads–Antidigoxin

Agarose acrobeads–antidigoxin were sterilized with 2.5 mrads of cobalt irradiation. In both in vitro and in vivo trials the beads showed no significant loss in digoxin removal ability during hemoperfusions.

Hemodynamic Conditions

Blood pressure remained within normal levels throughout the hemoperfusion procedure. Most probably this was a result of the relatively low volume of extracorporeal blood required by the system described.

The biocompatibility and hemodynamic data are typical of approximately 60 clinical trials with dogs and rabbits.

DESCRIPTION OF THE AGAROSE ACROBEADS

Photomicrography

Figure 4A is a photomicrography of the agarose acrobeads; mean diameter of these beads is 500–800 μm. Figure 4B is a cross-sectional photomicrograph of the beads showing the acrobeads encapsulated within the agarose matrix. Peripheries of two agarose acrobeads are depicted; mean diameter of the acrobeads is 0.2 μm.

Radial distribution of paraquat in the agarose acrobeads anti-paraquat is shown in Fig. 5. The uniform distribution of paraquat within the beads demonstrates the porosity of the beads; the antibody molecules easily penetrate the agarose matrix and are uniformly bound within the beads (*41*).

The deferoxamine and iron distribution in agarose acrobeads–deferoxamine before and after hemoperfusion trials was examined

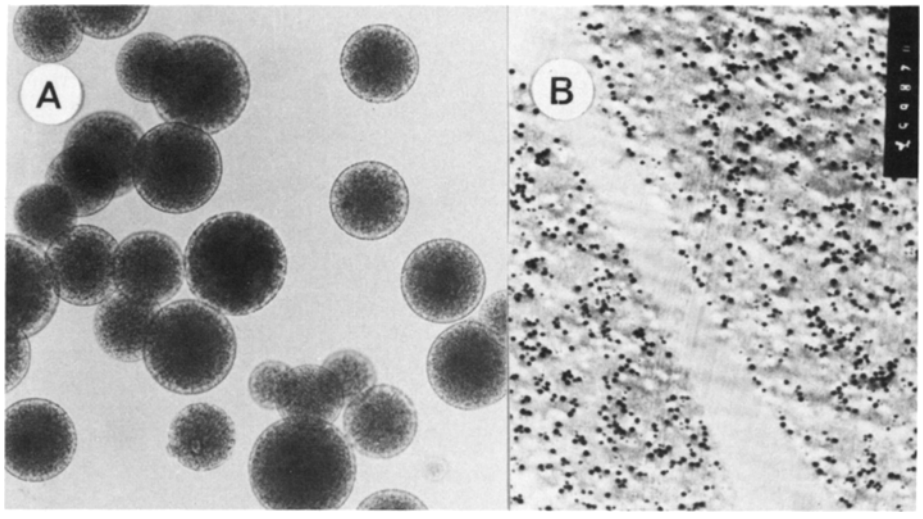

Fig. 4. (A) Photomicrograph of agarose acrobeads–ligand; diameters range from 500 to 800 μm. (B) Cross-sectional photomicrograph of the agarose acrobeads showing the acrobeads encapsulated within the agarose matrix; mean diameter of the acrobeads is 0.2 μm. Partial circumferences of two beads are depicted.

in the same manner. As expected, both the deferoxamine and the iron chelated are evenly distributed throughout the beads (*42*). Control beads lacking deferoxamine were free of iron under identical hemoperfusion conditions.

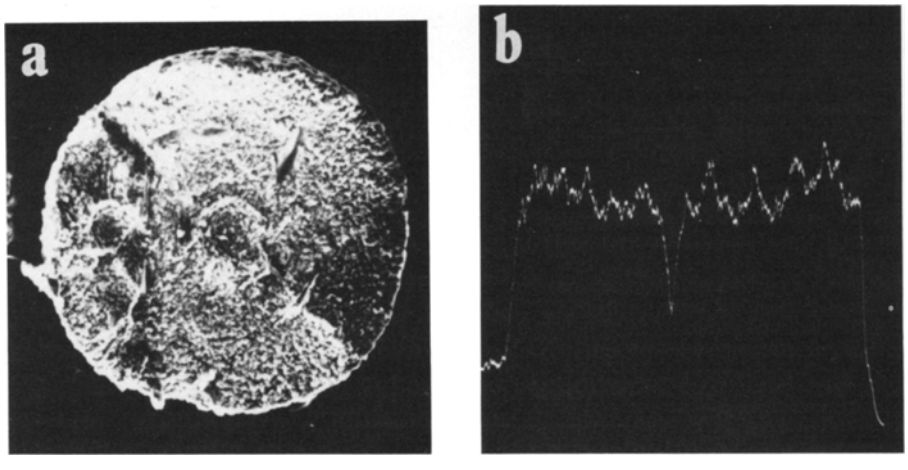

Fig. 5. (A) Radial distribution of paraquat in agarose acrobeads–antiparaquat conjugate after hemoperfusive removal of the intoxicant. (B) Line scan profile (LSP) of same. Agarose acrobeads–antiparaquat: paraquat were sectioned and reacted with lead chloride/ethanol. The paraquat content along the center of the bead was analyzed with a scanning electron microscope (SEM) with an energy dispersive analysis of X-rays (EDAX).

IN VIVO HEMOPERFUSION REMOVAL OF SPECIFIC ANTIGEN: THE MODEL SYSTEMS: DIGOXIN AND PARAQUAT

Detoxification of Digoxin from the Blood of Poisoned Animals

Cardiac glycosides are still the main therapy in the medical armamentarium for congestive heart failure and various cardiac arrhythmias. Unfortunately, since adequate blood digoxin levels for therapy are close to toxic levels, digitalis intoxication is a commonly encountered drug reaction in clinical medicine. A morbidity of 8–35%, with a mortality of 3–21%, in medicated patients has been reported (*46*). Additionally, digoxin has been used for self-poisoning; marked by an exceptionally high mortality rate. Since a specific antagonist for digitalis is generally unavailable, current therapy is directed toward the management of the clinical manifestations of toxicity and enhanced elimination of the body burden of digoxin. Unfortunately, severe intoxications may not respond to conventional measures and become life threatening. For these cases a method for significantly increasing the rate of removal or inactivation of digoxin is required.

Successful removal of digoxin from the circulation by hemoperfusion through coated charcoal or exchange resins has been reported (*47–50*). Hemodialysis is less than successful (*51,52*). The non-specific sorbents suffer from the disadvantage of low capacity and removal of needed biomolecules (*18*). The most exciting recent advance in therapy of digoxin intoxication is the infusion of antidigoxin antibodies or Fab antibody fragments to complex and inactivate digoxin (*53–56*). Renal excretion of the immune complexes is required to complete the therapy. The therapy we have developed and tested in animals combines the advantages of hemoperfusion and antidigoxin antibodies. The sorbent system, agarose acrobeads–antidigoxin is capable of specifically removing digoxin from the bloodstream of intoxicated animals while leaving cellular and soluble blood components not significantly affected. Hemoperfusion would be effective in a patient with an impaired or nonfunctional renal system. Clinical manifestations of digoxin intoxication, as evidenced by vomiting, diarrhea, tachycardia, and severe ECG changes, were ameliorated in the process. The dogs survived intoxications fatal to nonhemoperfused animals.

The time course of removal of digoxin during a typical hemoperfusion is depicted in Fig. 6. Digoxin was equilibrated into tissue compartments; life threatening arrythmias were recorded, and hemoperfusion therapy was initiated. In this trial the digoxin serum level decreased by more than 60% after approximately two blood volume flows through the column. The pre- and postcolumn differential was 3 ng/mL at termination of hemoperfusion, showing that the agarose

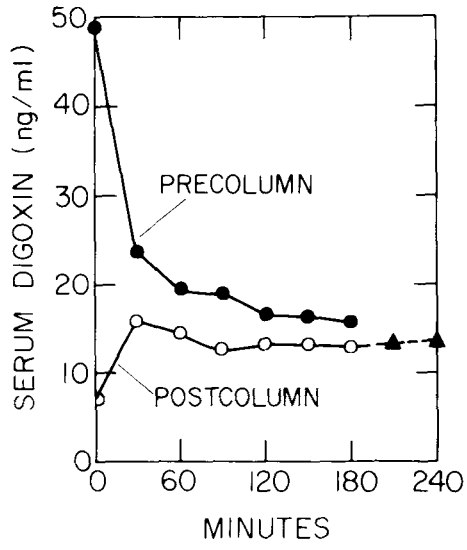

Fig. 6. Rate of removal of digoxin by hemoperfusion of the blood of an intoxicated dog through agarose acrobeads–antidigoxin. The dog received 0.09 mg digoxin/kg weight on each of three successive days. Hemoperfusion was begun after equilibration of the last dose. Dog wt = 25 kg. Blood flow rate = 120 mL/min. 25 g agarose acrobeads–antidigoxin in the column: ●, = serum digoxin content (prior to entrance into the column); ○, = serum digoxin content at exit from the column; ▲, = serum digoxin content of blood sampled upon cessation of hemoperfusion.

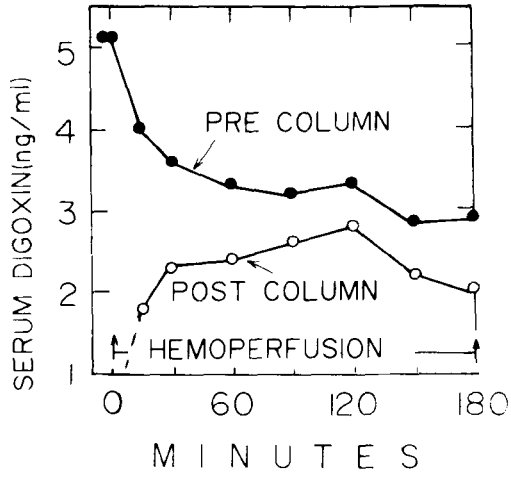

Fig. 7. Rate of removal of digoxin by hemoperfusion of the blood of a "low level" intoxicated dog through agarose acrobeads–antidigoxin. The dog received a single dose of 0.07 mg digoxin/kg 18 h prior to hemoperfusion. Dog w = 18 kg. Blood flow rate = 120 mL/min. 10 g agarose acrobeads–antidigoxin in the column. ●, = serum digoxin content (prior to entrance into column). ○, = serum digoxin content at exit from the column.

acrobeads still possessed adequate capacity for digoxin. The ECG pattern returned to normal and the dog survived the intoxication. No digoxin rebound in the blood was detected at 60 min after this trial; none was detected after 180 min in six trials in which it was measured 3 h after hemoperfusion.

Digoxin doses required to produce arrythmias in dogs are orders of magnitude above those in humans. Therapeutic levels for man are of the order of 2 ng/mL serum; intoxicating levels are above 3 ng/mL serum. Therefore, it was of interest to determine the efficacy of our system at lower body burdens of digoxin. Figure 7 shows a typical hemoperfusion trial with lower blood digoxin level and a similar, but smaller, column containing a lesser quantity of agarose acrobeads–antidigoxin. Serum digoxin content fell by some 40%. This would indicate the use of approximately 25 g columns containing agarose acrobeads of 5 mg antidigoxin/g beads for human emergencies.

Electrocardiogram (ECG) Tracings During Hemoperfusion Trials

ECG tracings obtained during the course of a typical hemoperfusion trial are shown in Fig. 8. Dramatically improved ECG changes are noted within one blood volume flow through the column. The preintoxication pattern is usually obtained after two blood volume flows through the column. Figure 9 contains ECG tracings of a similarly treated control dog hemoperfused with agarose acrobeads lacking antidigoxin. The dog did not survive the intoxication.

We might anticipate some level of rebound phenomenon following hemoperfusion, though none was seen either in the ECG pattern (Fig. 10) or in blood digoxin up to 3 h posthemoperfusion. We did not specifically measure digoxin content after 3 h, but did note that all dogs survived and were well indefinitely after any given hemoperfusion. This would imply that the level of rebound that might have occurred, but was not recorded, did not cause severe complications requiring further hemoperfusion.

Digoxin intoxication, manifested by vomiting, tachycardia, ECG changes (long runs of premature ventricular contractions progressing to ventricular fibrillation) and death in untreated animals was reversed during hemoperfusion through the agarose acrobeads–antidigoxin. Digoxin in the serum is rapidly removed by the beads during hemoperfusion. Digoxin bound to RBC receptors is removed slightly more slowly than that in the serum. Digoxin bound to other tissues (heart, kidneys) equilibrates with the serum component and is removed at an even slower rate (*49*). In any event, both in chronic and acute intoxication, sufficient digoxin is removed within two blood volume perfusions through the beads to bring the ECG from a potentially lethal state to a nonlife-threatening one. Under our experimental conditions the ECG pattern returns to the preintoxicated state within 2 h. The dogs were well and sur-

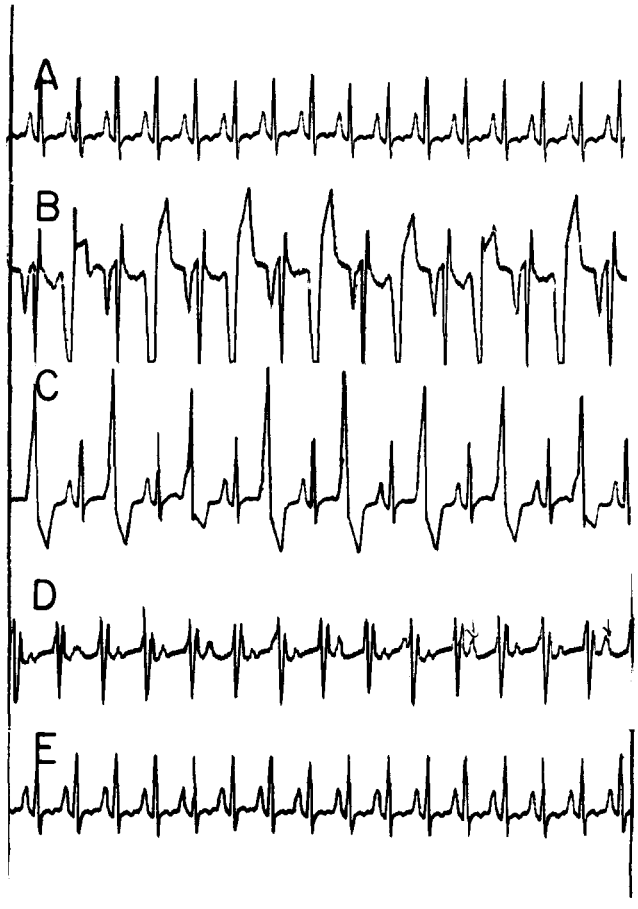

Fig. 8. ECG tracings during the course of hemoperfusion of a digoxin intoxicated dog. Dog wt = 24 kg. 30 g agarose acrobeads–antidigoxin in the column. Blood flow rate = 150 mL/min. The dog received 0.09 mg digoxin/kg body wt on three successive days. A. ECG prior to third dose, B. 45 min after third dose. Potentially lethal dysrhythmia noted. Hemoperfusion started 1 h after third dose, C. 20 min during hemoperfusion, D. 40 min during hemoperfusion, and E. 1 h during hemoperfusion. At 20 min after the start of hemoperfusion (C), the equivalent of one blood volume pass through the beads, great improvement appeared on the monitor. At 1 h of hemoperfusion the ECG pattern prior to intoxication was established (E).

vived indefinitely after hemoperfusion. Thus far, up to 27% of the total body burden of digoxin was removed, which exceeds the amount of digoxin in the serum. This and the therapeutic effect on the rhythm disturbances suggest that cell receptor bound digoxin was also removed during hemoperfusion. The agarose acrobeads–antidigoxin were not saturated during the trials, indicated by the pre/postcolumn digoxin differential.

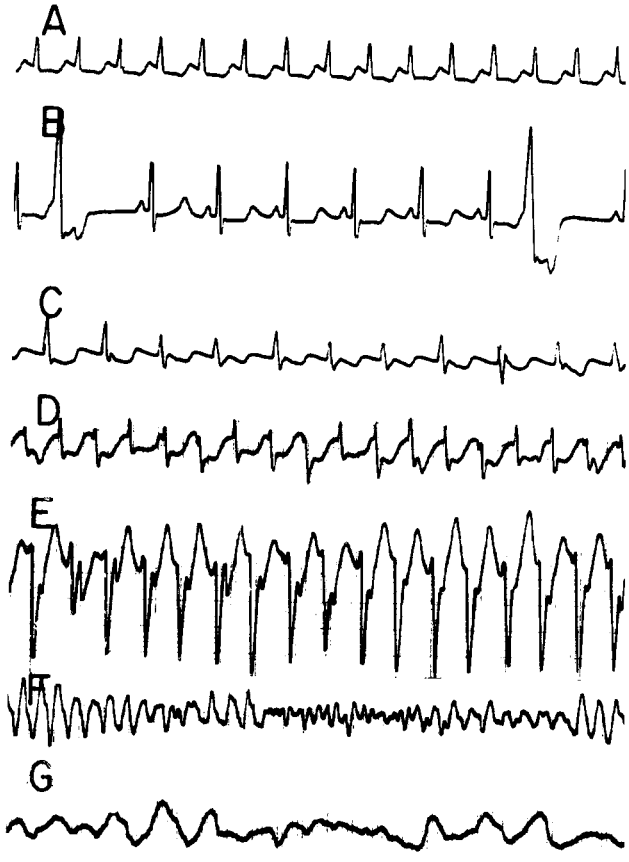

Fig. 9. ECG tracings during the course of a hemoperfusion control, in which the blood of a digoxin intoxicated dog perfused through beads lacking antidigoxn. Dog wt = 15 kg. 25 g agarose acrobeads in the column. Blood flow rate = 120 mL/min. Dog received 0.09 mg digoxin/kg body wt on each of three successive days. A. Prior to third dose, B. 10 min after third dose. Hemoperfusion began at 15 min after third dose, C, D, E, F, and G are at 15, 30, 90, 95, and 100 min during hemoperfusion, respectively. Malignant arrythmias appeared by 20–30 min, (C,D,E); terminating in ventricular fibrillation and death (F, G).

Dogs tolerate the procedure well and remain alive and healthy. Twenty-one dogs participated in 37 separate hemoperfusion trials. Control dogs (nontreated or managed with cardiopulmonary resuscitation and infusion of antiarrythmic agents or hemoperfused with beads lacking antidigoxin) died in ventricular fibrillation. Twelve dogs were used once, four were used twice, three were used thrice, and two were used four times. However, the ECG tracings usually returned to normal within 2 h in all these dogs, and the potentially lethal intoxication was reversed. In trials in which serum/body digoxin level had equilibrated, 50–95% of the serum was removed during hemoperfusion (*39*). In low-level intoxication trials, digoxin serum level was reduced by as much as

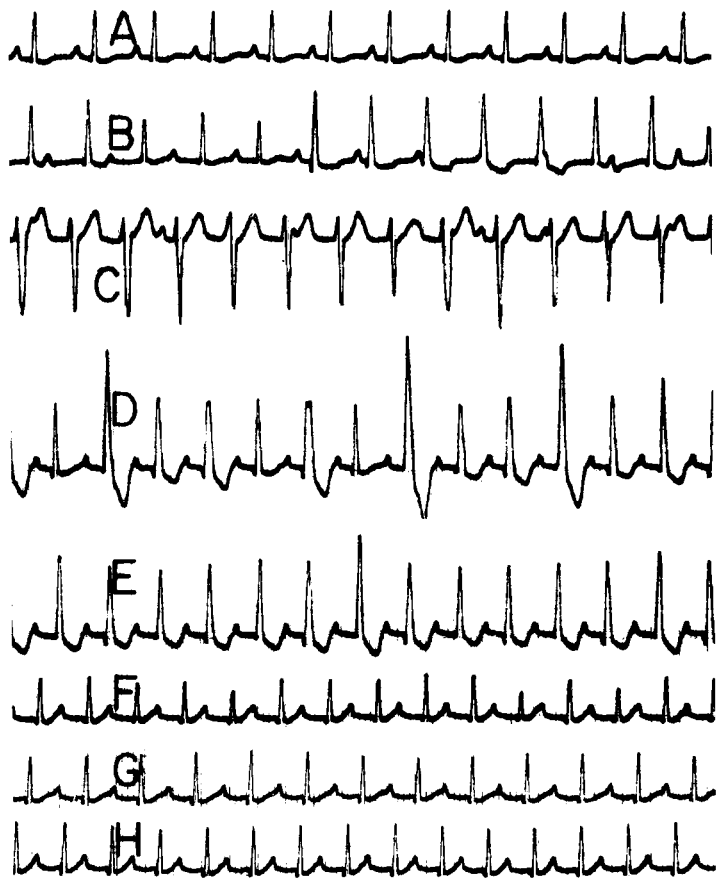

Fig. 10. ECG tracings during the course of hemoperfusion of a digoxin intoxicated dog. Dog wt = 25 kg. 25 g agarose acrobeads in the column. Blood flow rate = 120 mL/min. Dog received 0.09 mg digoxin/kg body wt on three successive days. A. Prior to third dose, B. 60 min after third dose, C. 70 min after third dose, D. 10 min during hemoperfusion, E. 20 min during hemoperfusion, F. 30 min during hemoperfusion, G. 40 min during hemoperfusion, normal sinus rhythm is noted at G, and H. 180 min after cessation of hemoperfusion; no rebound seen at 3 h after hemoperfusion.

80% (*40*). This value may be somewhat low, since, presumably, some of the total body digoxin equilibrated into the serum during hemoperfusion.

Agarose acrobeads–antidigoxin sterilized by cobalt irradiation possess the same level of digoxin complexing activity as do nonsterile beads. It bears repeating here, that the levels of digoxin required to intoxicate dogs are orders of magnitude higher than those obtained in humans in acute or chronic intoxication or upon suicide attempts. Thus, the agarose acrobeads–antidigoxin used in these trials should manage most cases of human digoxin intoxication.

The sytem described here combines the advantages of most of the current therapeutic measures for digoxin intoxication and is free of most

of the drawbacks. It is specific, has a relatively high clearance rate, and does not require priming with foreign blood or plasma. The system does not require the penetration into the bloodstream of any foreign substance, i.e., antibodies, fragments of antibodies, or immune complexes, and does not require renal excretion of complexes. Therefore, it should be safe in patients with congestive heart failure, diabetes, renal impairment, or other concommitant diseases. The beads do not affect the formed elements or soluble components of the blood, as reported with some other systems. Additives to the blood are not required. The poisoning levels of digoxin in humans are relatively low, thereby allowing small columns and minimal extracorporeal blood. The equipment is similar to that used in hemodialysis and related procedures and thus, should be readily available in most hospitals.

Detoxification of Paraquat from the Blood of Poisoned Animals

The herbicide paraquat plays an important role in bringing crops to market by decreasing crowding/competition for space and nutrients between the cash crop and weeds and reducing the labor required to weed fields. With proper precautions paraquat is a safe herbicide. However, accidental spray contamination, oral ingestion, or deliberate intake (self-poisoning or smoking 'tobacco' sprayed with paraquat) causes severe morbidity and mortality, ranging from 30 to 50% in Europe (*57*) to 90% in Japan (*58*). Detoxification of the herbicide from the blood as early after contact as possible is essential to reduce accumulation into the organs of the body, especially the pulmonary system. The recommended therapy currently in use is peritoneal dialysis or hemodialysis (*59,60*). Unfortunately, these procedures ae marginally effective at best (*61,62*). Hemoperfusion of poisoned blood through activated charcoal or nonionic exchange resins is equivocally effective (*14,41,58,63–65*). Hemoperfusion through fuller's earth is more effective than through encapsulated charcoal (*17*). Hemoperfusion through materials specific for paraquat, i.e., agarose acrobeads containing antiparaquat antibodies, should be more effective, as well as obviate the removal of important biocompounds noted with nonspecific sorbents.

Preliminary in vitro experiments in our laboratory with agarose acrobeads–antiparaquat demonstrated the efficacy of the system (*66*). The kinetics of removal of paraquat in hemoperfusion clinical trials with herbicide dosed animals are shown in Fig. 11. Under our experimental conditions, removal of paraquat from the blood is very rapid; 42% is removed within 10 min; 60% of the paraquat is removed within 2 h. Columns containing 33 g beads were used in these trials. For human intoxications, we calculate that bead volumes approaching those of commercialy available columns or larger will be required (*66*).

Charcoal and agarose encapsulated fuller's earth have a somewhat higher capacity than our system (*17,41*), although prolonged hemo-

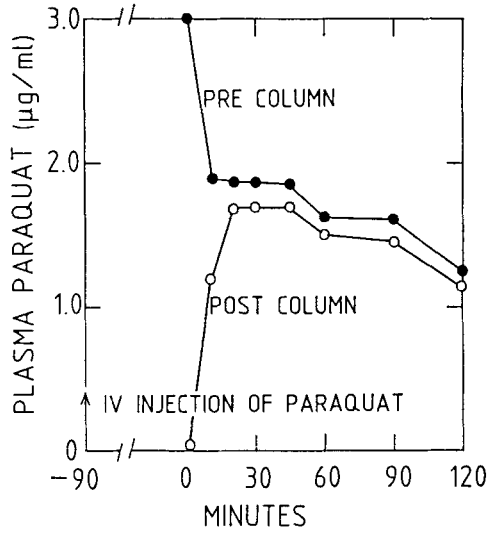

Fig. 11. Rate of removal of paraquat by hemoperfusion of the blood of an intoxicated dog through agarose acrobeads–antiparaquat. Dog wt = 9.5 kg. The dog received 2.2 mg paraquat/kg wt, iv, prior to hemoperfusion. Blood flow rate = 80 mL/min. 33 g agarose acrobeads–antiparaquat in the column; ●, = serum paraquat content (prior to entrance into the column); ○, = serum paraquat content at exit from the column.

perfusion times may be required. However, the agarose acrobeads have a much higher affinity and specificity for paraquat. Furthermore, infusion of acid citrate dextrose (ACD) postcolumn is required to decrease platelet depletion in the fuller's earth system.

We have demonstrated that the much higher affinity of the agarose acrobeads–antiparaquat accelerates the rate of removal of the herbicide from blood. Furthermore, the high affinity enables detoxification of patients whose plasma levels are low. There are indications that the greater affinity of our beads may aid in removing paraquat from tissue. Commercial sorbents are unable to accomplish this. Thus, one might envision tissue clearance over a period of time. Successive hemoperfusions might clear tissue of the herbicide. Further development is required to increase the capacity toward paraquat before human trials. This might be accomplished by increasing the amount of antiparaquat bound to the beads or by binding Fab fragments to the beads. It should be noted here that the order of magnitude of paraquat in the blood is some 1000 times greater than that found in digoxin poisoning.

REMOVAL OF SPECIFIC ANTIBODY

Autoimmunological Disorders

Autoimmunological diseases (symptoms are caused by antibodies produced against the patients own tissue) seem most amenable to help

TABLE 4
Representative Autoimmunological Disorders

Syndrome	Disorder
Rheumatic syndromes	
Systemic lupus erythematosis	Antibodies against nuclei and DNA
Rheumatoid arthritis	Articular inflammation
Polymyositis	Inflammatory disease of muscle and skin
Renal syndromes	
Glomerulonephritis	Antibodies against basement membrane
Endocrine syndromes	
Throiditis	Antibodies against thyroid tissue
Hypothyroid	Antibodies against thyroglobulin
Hyperthyroid (Graves)	Antibodies against TSH receptors
Renal insufficiency (Addisons)	Antibodies against adrenal cortex
Diabetes mellitus	Antibodies against pancreatic islets
Ovarian failure	Antibodies against corpus luteum
Other	
Myasthenia gravis	Antibodies against acetylcholinesterase receptors

by hemoperfusive therapy. Perhaps 100 autoimmunological syndromes have been described, many of which are well studied with advanced understanding of the molecular biology of the disease processes (*67*). A few of the better understood disorders are shown in Table 4.

The technique of plasmapheresis, that is, complete removal of the plasma of patients with syndromes ascribed to autoantibodies, has been employed for rheumatoid arthritis, myasthenia gravis, thyroiditis, glomerulonephritis, and so on with somewhat equivocal, but encouraging, results (*68*). However, this therapeutic modality removes all soluble blood components, requiring subsequent reinfusion of donor serum albumin or fresh frozen plasma with its attendant danger of hepatitis, AIDS, and other such problems. Alternatively, saline may be reinfused, leaving the patient deficient in needed antibodies and other factors. These dangers may be obviated with the use of hemoperfusion. In our case, the agarose acrobeads are fabricated to contain the antigen evoking the antibodies. The patients' blood is hemoperfused through the conjugated beads; the offensive antibodies selectively adsorbed.

Hemoperfusion is palliative; it should reduce the symptoms, and so on, but would have to be repeated periodically. It has been suggested that once the antibody burden is greatly diminished, chemotherapeutic agents would be added to the therapeutic regimen to eliminate the anti-

body forming lymphocytes. In any event, this therapy should decrease symptoms and make life bearable for these patients.

The Model System: Removal of AntiBSA with Agarose Acrobeads–BSA

We developed a model system in which rabbits were repeatedly immunized with BSA, such that greater or lesser blood vessels were obtained (*38*). Furthermore, we developed agarose acrobeads, the ligand of which was BSA bound through a spacer of polylysine glutaraldehyde (*27*). Circulating antiBSA antibodies were adsorbed to the conjugated beads during hemoperfusion (*27,38*). If required, antiBSA subsequently may be eluted quantitatively from the beads.

Kinetics of Removal of AntiBSA from Immunized Rabbits

Immunized rabbits were fitted with cannulas and coupled to the extracorporeal system. The case where the capacity of the quantity of agarose acrobeads–BSA used was exceeded is shown in Fig. 12. Forty-four percent of the antiBSA was removed in 30 min; 51% in 60 min; and the beads saturated within 105 min. In total, 323 mg of antiBSA were removed. It is not anticipated that a patient would bear such a large burden of antibody. However, in that rare case larger columns would be employed. The column used here contained 15 g beads.

A more typical example in which the column is operated within capacity is shown in Fig. 13. Sixty-two percent of the antiBSA was removed in 30 min; 78% in 60 min; 95% in 120 min; and 96% in 180 min.

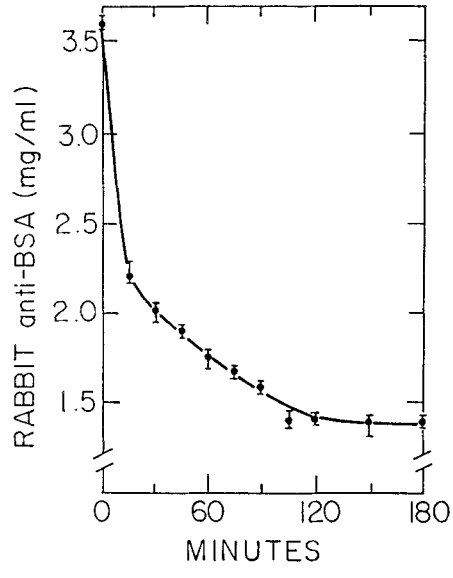

Fig. 12. Rate of removal of antiBSA from three immunized rabbits. Rabbit serum averaged 3.6 mg antiBSA/mL. Rabbit wts = 3.5–4.5 kg. 15 agarose acrobeads–BSA/column. Blood flow rate = 15 mL/min. Quantity of antiBSA exceeded the capacity of the column.

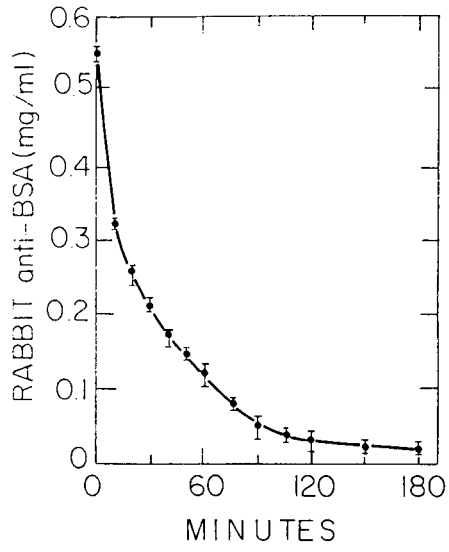

Fig. 13. Rate of removal of antiBSA from four immunized rabbits. Rabbit serum averaged 0.55 mg antiBSA/mL. Rabbit wt = 3.5–4.5 kg. 15 g agarose acrobeads–BSA/column. Blood flow rate = 15 mL/min.

In an extended series of rabbit trials similar to these, we demonstrated that the agarose acrobeads–BSA are indeed capable of removing specific antibodies while leaving the blood components essentially unaffected (*37*). This is our model system presaging an attack on several autoimmune disorders.

The Model System: Removal of Circulating Immune Complexes

Immune complexes (IC) have been demonstrated in patients with a variety of autoimmune, infectious, and malignant syndromes; for example, DNA/antiDNA IC in patients with systemic lupus erythematosis (*69*), acetylcholine receptor/antiacetylcholine receptor IC in patients with myasthenia gravis (*70*), IgG/antiIgG IC in patients with rheumatoid arthritis (*71*), and IC in patients with persistent hapatitis B virus (*72,73*) and various malignant disorders (74,75). Standard methods have been used to isolate and characterize the IC, i.e., protein A Sepharose (*76*), binding to Raji cells (*77*), and binding to rhematoid factor coated tubes (*78,79*); recently, C1q and conglutinin were employed in solid-phase systems (*73,80*). We have employed C1q as an affinity immunoadsorbent for removal of circulating IC (*40*) thus far in an in vitro system. We hope to scale up the isolation of C1q for use in hemoperfusive therapeutic systems.

In in-vitro affinity chromatography trials with agarose acrobeads–C1q we have removed 80% of the BSA–antiBSA IC in three perfusions of plasma-containig IC through the column (Table 5). Similar perfu-

TABLE 5
Binding BSA/antiBSA Immune Complexes (IC) to C1q[a]

Passage number	Radioactivity bound, cpm	Radioactivity eluted, cpm	Recovery, %	IC eluted, mg protein
1	34,500	24,260	70	0.50
2	26,600	20,800	78	0.48
3	25,400	21,100	83	0.40

A solution containing BSA/antiBSA IC (1.6 mg, 90,000 cpm) was passed through agarose acrobeads–C1q (2 mg C1q) three times. The column was regenerated (bound IC were eluted) after each passage. Two milligrams C1q bound approximately 0.5 mg IC/pass. The overall IC recovery according to protein determination of the pool of eluted material was 1.38 of the 1.6 mg passed through (86%). Recovery according to radioactivity was approximately 80%.

sions with plasma-containing hepatitis B virus surface antigen–HBVsAg antibodies IC removed a lesser percentage. The purity of the IC removed was ascertained by gel electrophoresis.

REMOVAL OF TOXIC CATIONS FROM BLOOD

The Model System: Removal of Iron from Poisoned Animals

The pathology, morbidity, and mortality of acute and chronic iron poisoning are well documented (*81–83*). Unfortunately, there is no physiologic route for the elimination of excess iron in humans (*84*). Chronic iron overload occurs in a variety of diseases in which the administration of parenteral iron is necessary (thallessemia, aplastic anemia) (*85*) or through increased intestinal absorption of dietary iron (hemochromatosis) (*86*). Acute iron poisoning from accidental ingestion of iron tablets are responsible for an estimated 2000 hospitalizations annually in the United States (*87*). In acute iron overload, the iron transport system is overwhelmed, resulting in free, toxic iron [in excess of the total iron-binding capacity (TIBC)] (*88*). This generates highly reactive free oxygen radicals, causing lipid peroxidation and membrane damage (*89*). In chronic iron overload the excess iron is deposited mainly in the spleen, liver, and heart, causing widespread damage (*90*).

The therapy of phlebotomy cannot be employed for hemosiderosis or acute iron poisoning (*91*). The modern treatment of acute iron overload (iron poisoning) is the slow, subcutaneous iv drip of deferoxamine in tandem with conventional life supporting measures (*92–94*). The feroxamine formed is eliminated in the urine. Unfortunately, deferoxamine (a) has a short serum half-life (*95*), (b) when given via the oral route is both inefficient (*88*) and somewhat toxic (*93,95*), and (c) is expensive. Additional problems arise in cases of renal failure because the toxic feroxamine cannot be eliminated by urinary excretion (*96,97*).

Hemodialysis is an ineffective therapeutic modality because much of the excess iron is nonspecifically bound to albumin and cannot cross the dialysis membrane. Nonspecific sorbents are likewise ineffective. Ramirez and Andrade (*98,99*) bound deferoxamine to polyacrolein powder, but their sorbent system failed because it was relatively impervious to liquid flow. A specific hemoperfusive system in which the agarose acrobeads are coupled to deferoxamine is both effective and usable in patients with renal failure. Excess iron, along with its nonspecific protein carrier, can easily enter the agarose acrobeads–deferoxamine. The beads may be crosslinked and sterilized in an autoclave (*100*) without altering their chelating ability.

HEMOPERFUSIVE DETOXIFICATION OF IRON THROUGH AGAROSE ACROBEADS–DEFEROXAMINE

Normal serum iron levels fluctuate narrowly within a range centered about 1 ppm. Levels of 5–10 ppm are dangerous. Levels above 10 ppm are potentially lethal, requiring emergency, sometimes heroic, measures.

The rate of removal of iron in dosed whole blood to well below the TIBC is shown in Fig. 14. Potentially lethal or dangerous levels of iron were brought down below the TIBC level within 1 h.

Figure 15 illustrates the rate of removal of iron during a hemoperfusion trial in a severely, acutely intoxicated dog. Up to 40% of an extremely high level of iron was removed. Subsequent hemoperfusions would be required to bring the levels below the danger level. But this may still be better than the standard therapy of deferoxamine infusion.

Hemoperfusion trials with "chronically" iron overloaded dogs showed a decrease in serum iron levels of some 29% (Fig. 16).

In all of these hemoperfusion trials, both the formed elements of the blood, as well as the usual battery of soluble components determined in the standard SMA 12 analysis, were not significantly altered.

Usually, the iron in serum is tightly bound to its transport protein (transferrin); tissue iron is bound to the iron storage proteins (ferritin and hemosiderin). Transferrin is normally only one-third saturated, so tht the TIBC is about 3 ppm. Deferoxamine cannot remove iron from transferrin; thus, in our system, the lowest theoretical iron level attainable is the TIBC. However, the greater the degree that transferrin is saturated, the easier iron is removed from it.

Current treatment of chronic iron overload is, as mentioned, the continuous subcutaneous administration of deferoxamine. By this treatment, about 40–100 mg iron is secreted in the urine daily, depending on the dose. In our case, because of the low affinity of the sorbent and the failure of the "chronic" iron overload model, much smaller amounts were removed. Deferoxamine possesses high affinity for iron (*99*). Unfortunately, during binding of deferoxamine to acrobeads there is a great

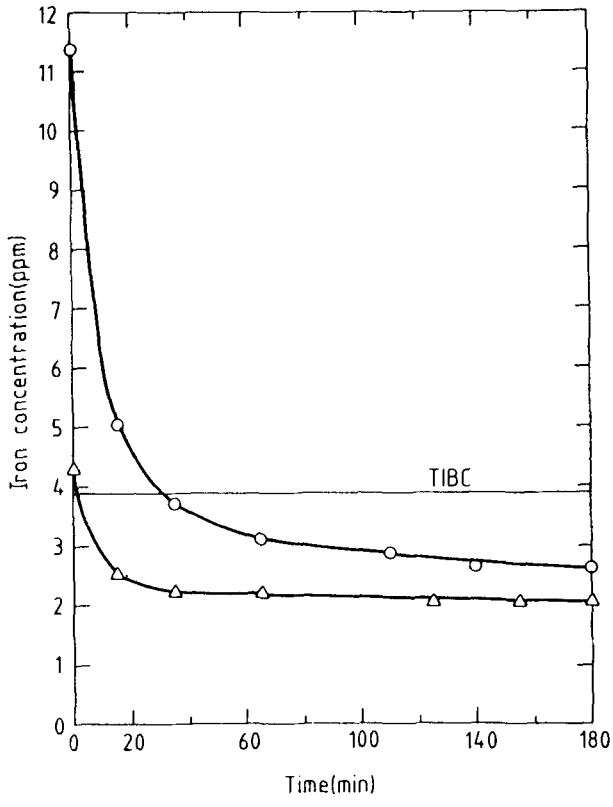

Fig. 14. Rate of removal of iron from whole blood (in vitro). Resevoir contained 250 mL heparinized goat blood. Blood flow rate = 35 mL/min. 15 g agarose acrobeads–deferoxamine/column.

loss of affinity, although the capacity remains high (*100*). However, theoretical considerations do not predict a large amount of iron being removed, even with a better sorbent. This is because of the small amount of iron present in the serum at any time. The present day treatment is superior because the administered deferoxamine chelates not only the labile pool of free cytoplasmic iron in the cells, but also the reticuloendothelial and parenchymal iron. Hemoperfusion may be a useful method to remove iron in acute iron intoxication.

FUTURE PLANS

In the immediate future are the human clinical trials for hemoperfusive removal of digoxin in severely digoxin-intoxicated patients. In the near term, specific autoimmune syndromes will be examined as candidates for palliative therapy employing both agarose acrobeads-specific antigens to remove specific antibodies and agarose acrobeads–C1q or –protein A to remove immune complexes. Further-

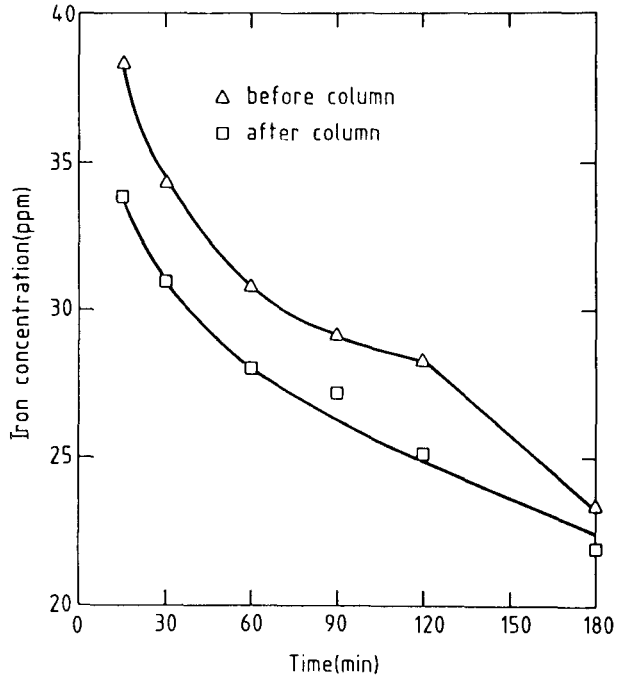

Fig. 15. Rate of removal of iron by hemoperfusion of the blood of an "acutely" intoxicated dog through agarose acrobeads–deferoxamine. The dog received 60 mg iron (as ferric chloride) dissolved 100 mL of his plasma, iv. Dog wt = 17 kg. Blood flow rate = 120 mL/min. 50 g agarose acrobeads–deferoxamine/column: △, plasma iron content prior to entry into the column; □, plasma iron content at exit of column.

more, we are producing antiLDL antibodies to fabricate agarose acrobeads–antiLDL to remove LDL and LDL cholesterol from the bloodstream of hypercholesteremic animals. Longer-term plans include the use of agarose acrobeads–antichemotherapeutic agents for the "rescue" technique in cancer chemotherapy. Thus, patients may be treated with larger doses of chemotherapeutic agents for short intervals, and the excess agent removed by hemoperfusion. Finally, we will examine an agarose acrobeads–antiendotoxin system to remove endotoxins in patients with endotoxic shock. Limitations in the use of this general technique are set only by the investigator.

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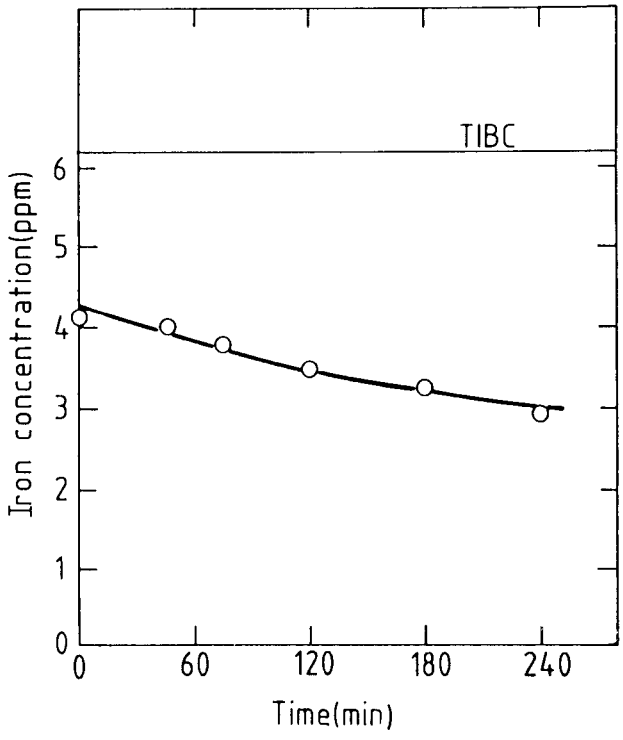

Fig. 16. Rate of removal of iron by hemoperfusion of the blood of a chronically intoxicated dog through agarose acrobeads–deferoxamine. The dogs received a final load of 1 g elemental iron/kg body wt by iv injection of Imferon 4 times/wk for 3 mo. Dog wt = 12 kg. Blood flow rate = 120 mL/min. 50 g agarose acrobeads–deferoxamine/column.

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