FUNCTION OF THE RETICULOENDOTHELIAL SYSTEM

II. PARTICIPATION OF A SERUM FACTOR IN CARBON CLEARANCE*

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The necessity for there to be some serum factor(s) for optimal phagocytosis by free vertebrate cells has long been recognized. Indeed, the presence of serum is essential for the phagocytosis of certain bacteria and inert colloidal particles (1–3). Several different serum components have been implicated in this process including; complement (3–5), properdin (6), specific antibody (7, 8), some form of heat-labile, and heat stabile factor (9), and in the case of starch phagocytosis an alpha-1 and beta-globulin (10). However, the nature and function of serum factors required for phagocytosis *in vivo* and their relationship to reticuloendothelial blockade has not yet been clearly defined.

The study of phagocytosis *in vivo* has depended on measuring the rate of clearance from the blood of foreign or altered autologous particulate substances, and an alteration in the rate of clearance has been observed following prior injection of identical or similar material (11). This reduction in the rate of clearance has been termed "reticuloendothelial blockade" and it has been suggested that it results either from saturation of the phagocytic cell (12) or from depletion of available serum opsonins (13–15). Jenkin and Rowley reported that treating carbon with serum prior to injection enhanced the clearance and, furthermore, prevented the "blockading effect" of prior carbon administration (13). Recently, however, Biozzi and coworkers (12), repeating these experiments, concluded that serum factors neither altered the slope of the clearance curve nor removed a carbon-induced "blockade" of the reticuloendothelial system; they concluded that saturation of the phagocytic cell was the principal mechanism involved in the reduced rate of carbon removal. Resolution of this question of whether an altered clearance of carbon might reflect changes in available serum opsonin invited the present study.

In the experiments reported here advantage was taken of the observation that denatured albumin injected while carbon is circulating depresses the rate of colloidal carbon clearance in a regular and predictable way. In a previous publication we reported that quantitative relationships exist between the dose of albumin and the onset and duration of carbon clearance inhibition (16). Moreover, the gelatin content of the carbon preparation was found to prolong the

duration of inhibition to a given albumin dose. Since one effect of gelatin is to render the particle more stable in the blood it seemed reasonable to explore the possibility that the relationships found between albumin and carbon might represent a competition between the particles for an available serum factor. Such a serum factor has now been found to be present which, when adsorbed onto carbon *in vitro* can prevent the denatured albumin from reducing the rate of clearance of the carbon particles *in vivo*.

Materials and Methods

Clearance studies were performed on Sprague-Dawley rats anesthetized with intraperitoneal nembutal. Rat serum was obtained from several animals and pooled; it could be stored at −5°C for several weeks without loss of activity. The carbon preparation C-11-1431a was obtained from John Henschel and Co., Inc., New York, and was used as supplied without prior centrifugation or gelatin addition. Heat-aggregated bovine albumin was prepared as previously described (16, 17).

Unless otherwise indicated a standard dose of carbon (8 to 10 mg/100 gm of body weight) and denatured albumin (10 mg/100 mg of body weight) was used. This dose of carbon was selected to yield a sufficiently fast rate of carbon clearance so that any inhibition in the clearance rate due to denatured albumin injection would be readily apparent. The dose of denatured albumin was then adjusted so as to give a consistent reduction in the rate of carbon removal of sufficient duration as to be readily demonstrable. This dose of denatured albumin lies in the range where the duration of inhibition of carbon clearance is exponentially related to the dose of albumin administered (16). In each set of experiments the denatured albumin was tested daily to be sure that it was producing the desired inhibitory effect. Each individual experiment, of course, had its own control for the rate of carbon clearance measured as the rate of carbon removal prior to the injection of denatured albumin.

Extracts of rat serum were prepared using barium sulfate adsorption. The temperature of the serum was adjusted to 5°C and the barium sulfate (Allied Chemical Corporation, New York) was then added slowly with constant mixing as 4.6 gm of barium sulfate to 100 ml of serum. After mixing for 30 minutes the barium sulfate was removed by centrifugation at 5000 R.C.F. for 10 minutes. The pellet was washed twice with 0.15 M sodium chloride and then extracted in the cold for 45 minutes with 0.08 M sodium citrate, pH 7.4, in a volume equal to ½ the initial volume of serum. Barium sulfate was removed by centrifugation (45 minutes at 20,000 R.C.F.) and the eluate was dialyzed against 0.02 M phosphate and 0.065 M sodium chloride pH 7.4. Following concentration by pervaporation or ultrafiltration and redialysis against Hanks' balanced salt solution (or 0.02 M phosphate and 0.15 M sodium chloride at pH 7.4), the extract was recentrifuged at 10,000 R.C.F. for 45 minutes prior to use. The extracts were stored at 5°C and the protein concentration in each extract was determined using the ultraviolet method of Waddel (18).

The adsorption of serum proteins onto carbon was performed at 5°C using constant slow mixing with a magnetic stirrer for exactly 1 hour after addition of the commercial carbon to either the rat serum or the barium sulfate extract of rat serum. The quantity of carbon used was found to be a critical factor in relation to the protein content of the serum or extract. It was found that the weight ratio of carbon to protein must be maintained at less than 0.5 for serum and 1.5 for the barium extract. Inconsistent and often negative results were obtained when these ratios were exceeded. Following incubation the carbon concentration was determined spectrophotometrically prior to being injected into the animals. The pH of the mixture was maintained in the range of 7.2 to 7.8 and the final concentration of carbon did not exceed 18 to 20 mg per ml. The carbon-protein solution was injected cold and was not warmed to body temperature prior to use.

EXPERIMENTAL OBSERVATIONS

Heat Aggregated Albumin Inhibition of Carbon Clearance.—The rate at which a given carbon load is removed from the blood can be altered by the injection of heat-aggregated albumin during the course of carbon clearance. A typical curve depicting carbon clearance inhibition by denatured albumin is observed in Fig. 1. The initial part of the curve represents the clearance rate $(K-1)$ of the carbon prior to the introduction of denatured albumin and serves as a control. As soon as we can measure, following the introduction of denatured albumin, the clearance rate slows but does not reach a value of zero. Following this period of inhibited clearance, the curve abruptly resumes its normal course
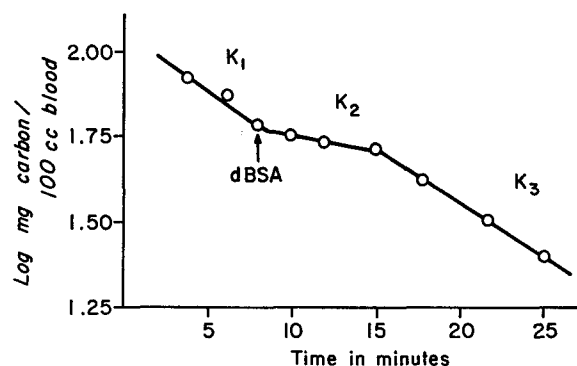
Fig. 1. A typical carbon clearance curve depicting the effect of an injection of heat denatured albumin (abbreviated dBSA) on carbon clearance. Note that three different phagocytic indices (K values) are associated with different portions of the clearance curve.

as seen at $K-3$ which also serves as a control, in this case for postinhibition of carbon clearance. The duration of depressed clearance $(K-2)$ has previously been shown to be dependent on the amount of albumin injected, the depth of inhibition being independent of the albumin dose (16). When 10 mg of denatured albumin are used to inhibit the clearance of 10 mg of carbon, the duration of inhibition lasts for an average of 6.2 minutes and causes a depression in the rate of clearance from a K_1 of 0.036 to a K_2 of 0.011. Therefore, this dose of carbon and albumin allows ready discernment of the inhibitory effect of albumin on the rate of carbon clearance and yet the duration of inhibition is not so prolonged but that a complete picture of the continuous change in the intravascular carbon concentration, including the postinhibition control K_3, is obtained.

Demonstration of a Serum Factor in Heat-Aggregated Albumin Inhibition of Carbon Clearance.—If a competition between albumin and carbon for a serum factor is responsible for the inhibition observed in the carbon clearance when

denatured albumin is injected, then treatment of the carbon particles with serum prior to injection might be expected to prevent the inhibition.

To test this possibility, carbon was incubated with homologous pooled rat serum for one hour at 5° C. Following intravenous injection into animals it was found that the carbon so pretreated with serum was removed at the same rate as the untreated carbon. However, when denatured albumin (10 mg/100 gm of body weight) was injected and the rate of clearance of carbon pretreated with
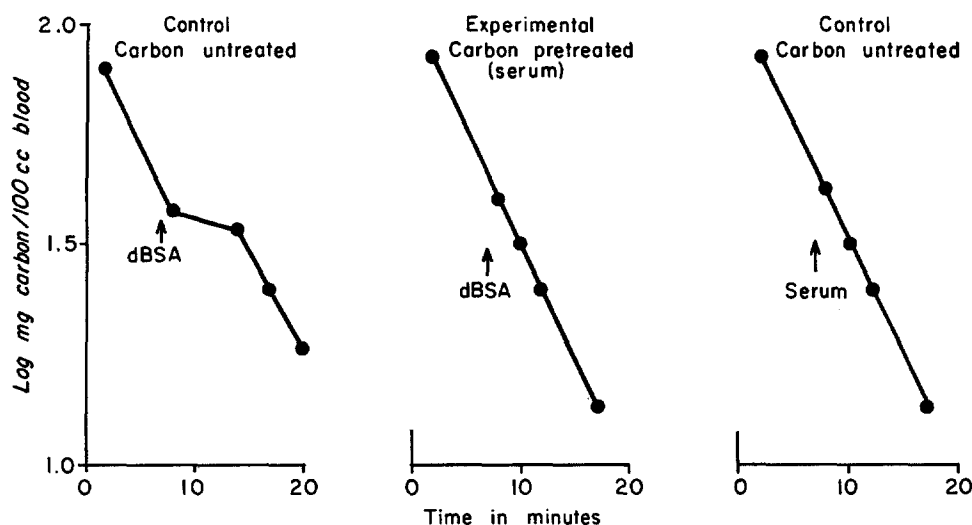

FIG. 2. The effect of serum pretreatment of particles *in vitro* on the rate of carbon clearance inhibition *in vivo* by denatured albumin (dBSA). A control curve is shown on the left illustrating the inhibition of carbon clearance by injection of dBSA. In the center is a representative curve of the effect of carbon pretreatment with serum; note, there is no inhibition when dBSA is injected. On the right is a control curve illustrating that the injection of serum alone has no effect on the rate of clearance.

serum was examined, it was found that the clearance of the treated particles was no longer depressed (Fig. 2). Moreover, serum alone injected in a dose of up to 1.5 ml/100 gm of body weight had no effect on the rate of carbon clearance.

Adsorption and Elution from Carbon of a Serum Factor Active in Preventing Denatured Albumin Inhibition of Carbon Clearance.—Since pretreatment of carbon with serum produces the effects described, the question arises as to whether serum contains an adsorbable material present in limited amount which is responsible for these phenomena. Adsorbed serum was prepared by suspending carbon in serum at 30 mg/ml. After removing the carbon by centrifugation at 25,000 R.C.F. for 30 minutes the serum supernatant was tested for its ability to protect carbon clearance against denatured albumin inhibition.

By repeated adsorptions it was possible to remove from the serum whatever is responsible for counteracting the effect of denatured albumin on carbon clearance (Table I). When 1 ml of serum was adsorbed with 30 mg of carbon, it still protected against albumin inhibition. When this serum supernatant was adsorbed with an additional 30 mg of carbon its protective ability was present in some but not in all animals. Following a third adsorption with 30 mg of carbon (total 90 mg carbon/ml serum) the serum no longer protected against albumin inhibition. If instead of repeated carbon adsorptions a single adsorption was employed, it was found that 60 mg of carbon per ml of serum was sufficient to remove all the material in serum responsible for protection against denatured albumin inhibition of carbon clearance.

TABLE I

Effect of Adsorption of Serum by Colloidal Carbon in Vitro on Action of Denatured Albumin on Carbon Clearance in Vivo

Protective ability is indicated as (+) when the carbon pretreated with serum is *not* inhibited by injection of denatured albumin.

Carbon per ml serum	Successive adsorptions	Total carbon	Protective ability
mg		*mg*	
30	1	30	+
30	2	60	±
30	3	90	−
60	1	60	−

Because carbon was found to deplete the serum of the factor responsible for preventing inhibition in carbon clearance by denatured albumin, the next step was to see if the protein adsorbed onto the carbon could be eluted from the carbon with preservation of its biologic activity. For this purpose the carbon was first purified by boiling in 10 N sodium hydroxide in order to hydrolyze any contaminating protein present in the carbon preparation. The carbon was next repeatedly washed in 0.15 M sodium chloride, and then added to the serum in an amount sufficient to insure that the unadsorbed serum supernate after centrifugation had lost its biological activity. (The carbon that had been boiled in 10 N sodium hydroxide could be easily removed from serum by low speed centrifugation at 2000 R.C.F. for 15 minutes.) Following centrifugation the carbon pellet was washed twice with 0.15 M sodium chloride to remove any non-adhering protein and subsequently extracted with 0.6 M sodium chloride at room temperature. Protein estimates by the Folin-Ciocalteu procedure (19) revealed that an average of 1.9 mg of serum protein per ml of serum had been adsorbed onto the carbon. Following dialysis and concentration by pervaporation the extracted protein was tested for biologic activity. When adsorbed onto Gunther-Wagner carbon (carbon/protein ratio of 1.0) and injected into animals, the treated car-

TABLE II

Materials which when Added to Serum Adsorbed the Factor Responsible for Preventing Denatured Albumin Inhibition of Carbon Clearance

Activity is considered + when clearance of carbon pretreated with serum extract eluted from adsorbing agent is not depressed by injection of denatured albumin.

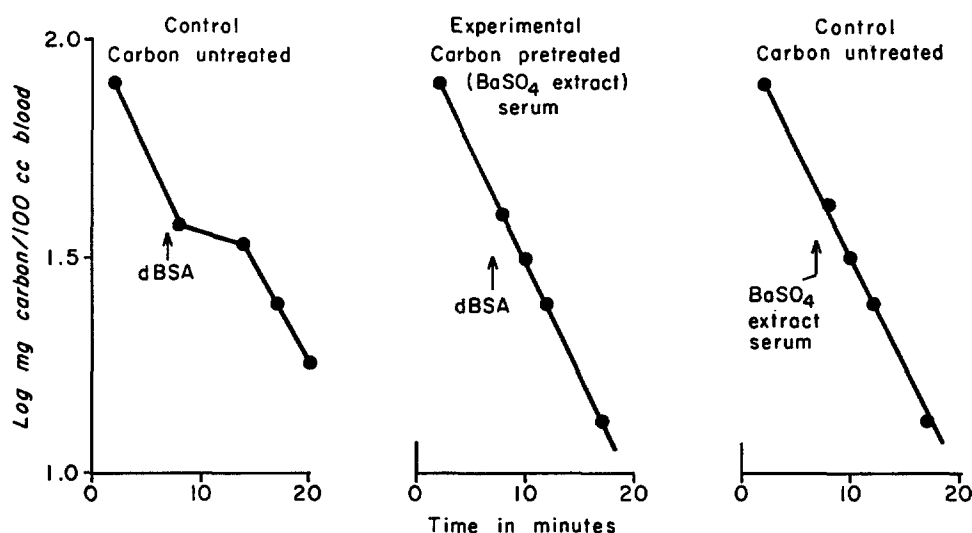| Adsorbing material	Eluting agent	Activity
Carbon (G-W ink)	0.6 M NaCl	+
Norite	0.6 M NaCl	+
Barium sulfate	0.08 M Na citrate	+
Bentonite	?	?

FIG. 3. The effect of a barium sulfate extract of serum on the rate of clearance of carbon particles and their inhibition by denatured albumin (dBSA). The curve on the left is a control curve illustrating the inhibiting effect of an injection of dBSA during the course of carbon clearance. In the center is a representative curve of carbon pretreated *in vitro* with a barium sulfate extract of serum; note, its rate of clearance is *not* inhibited by injection of dBSA. The curve on the right illustrates that injection of the barium extract during carbon clearance has no effect on the rate of clearance.

bon was cleared at the same rate as untreated carbon. In contrast to the untreated carbon, however, its rate of clearance was *not* depressed by injection of denatured albumin.

Barium Sulfate Adsorption Method for Initial Extraction of the Serum Factor(s).—The use of carbon to absorb serum is cumbersome. Therefore an alternative method of extracting the active material from serum was sought and sev-

eral different adsorbing agents were tried (Table II). Of these barium sulfate was the most promising being a relatively clean starting material and easy and efficient to use.

Adsorption of serum with barium sulfate using the procedure outlined above (see Methods) will deplete the serum of whatever material is responsible for preventing denatured albumin inhibition of carbon clearance. Moreover, the proteins adsorbed onto barium sulfate can be recovered by elution with sodium citrate and subsequently concentrated. The amount of protein recovered from the barium sulfate averaged 1.6 mg per ml of serum. When the material obtained from barium sulfate adsorption of serum was added to the commercial carbon preparation *in vitro*, it prevented the effect of denatured albumin *in vivo* (Fig. 3).

DISCUSSION

Previous studies concerned with the measurement of carbon clearance have emphasized a cellular limitation in the capacity to clear carbon deduced from the hyperbolic nature of the clearance curve and the reduced rate of clearance with large or multiple doses. Reticuloendothelial blockade was thought to represent the diminished phagocytic capacity of a cell as it became "saturated" with particulate material (11, 12). Recovery from such a blockade was viewed as resulting from cellular hyperplasia evidenced by the increased weights of the liver and spleen and the increased DNA synthesis in the liver following injections of colloidal materials (20, 21). Such a cellular regeneration theory, however, could hardly explain the rapid recovery of clearance rate when two colloids are simultaneously offered to the reticuloendothelial cells. Nor could "saturation" explain the low threshold of competing colloid necessary to elicit the blockade.

Exactly how denatured albumin functions when injected during carbon clearance is not clear. Four alternative possibilities suggest themselves; (a) the phagocytic cell becomes "saturated" or filled with ingested material and is therefore not able to ingest particles at its usual rate, (b) the denatured albumin binds onto the already coated surface of the carbon and thus retards its phagocytosis by the reticuloendothelial cells, (c) denatured albumin and carbon compete for available phagocytic sites on the cell, or (d) the aggregates of heated albumin compete for a serum component(s) necessary for maximal rate of carbon particle clearance, leaving the "naked" carbon without an adequate "opsonizing" coat.

The possibility that the phagocytic cell becomes "saturated" seems unlikely for the case of denatured albumin-carbon inhibition, in view of the fact that denatured albumin only temporarily retards the clearance and the retardation rapidly disappears. The observation that some serum proteins do adsorb onto the carbon particle *in vitro* and that such adsorption prevents depression of

carbon clearance by albumin *in vivo* suggests that the carbon particle surface is in some way altered so as not to interact with denatured albumin. Such an interaction between denatured albumin and carbon could be envisioned as a competition for phagocytic sites, albumin coating of the carbon particle surface, or as competition for some as yet unidentified serum component necessary for maximal rate of carbon phagocytosis. If the serum factor operates primarily as a promoter of phagocytosis, then the failure of the phagocytic rate to increase in response to coated particles suggests that sufficient serum factor is normally present to afford maximum rate of clearance in the doses used.

The exact role of the serum coat is not obvious from these experiments. It does seem clear, however, that some factor is present which participates in colloid clearance *in vivo* and that its reduction or absence could result in impaired reticuloendothelial function. The following evidence supports this conclusion; (a) the rapid onset and rapid return to normal of the depressed carbon clearance rate by denatured albumin precludes the occurrence of cellular regeneration during the course of the experiments, (b) the clearance of carbon coated prior to injection with barium sulfate extract of serum is not inhibited by injection of denatured albumin, and (c) the factor in serum responsible for the protection against denatured albumin inhibition is limited in quantity and can be selectively absorbed from serum by a sufficient amount of carbon. In fact, the material adsorbed on carbon and responsible for preventing denatured albumin inhibition appears to represent only a very small fraction of the total serum proteins.

The participation of a serum factor in intravascular clearance has been suggested by several authors. Thus clearance rates have been related to changes in gamma-globulin (22) and in serum titers of complement (5) and antibody (15, 23). Although studies relating clearance to antibody or complement usually refer to bacterial clearances, there is some evidence that serum proteins may also participate in the clearance of inert colloids as well. In this regard, the reported finding by Murray (15) of a circulating agglutinin to gelatin is particularly pertinent because many of these inert colloids are gelatin stabilized. Moreover, this same author has reported that the degree and duration of reticuloendothelial blockade to gelatin-stabilized colloidal gold induced by the same colloid was directly related to the circulating level of gelatin agglutinins and he suggested that opsonins rather than saturation of the phagocytic cell provided the limiting factor in the clearance rates. This is particularly interesting in relation to the present study on serum factors and carbon clearance when it is appreciated that the rate of clearance of colloidal carbon is itself decreased as the content of gelatin increases and that with increasing gelatin in the carbon preparation there is an exponential prolongation in the duration of inhibition to a given albumin dose (16). Whether the factor that adsorbs onto barium sulfate is indeed a gelatin agglutinin or is some other component of serum is not apparent from the present study and must await further clarification.

Jenkin and Rowley (13) have previously reported that a carbon-induced blockade of the reticuloendothelial system could be eliminated if the carbon test dose was treated with serum; this has been denied by Biozzi *et al.* (12). The latter authors maintain that carbon induced blockade results from cell saturation and that the rate of carbon clearance is not altered either by the injection of serum during carbon clearance or by treating the carbon with serum. Our observation that the level of the factor normally present in rat serum varies and that the ratio of carbon to serum is critical may not have been appreciated by these authors and may in part help to explain their divergent conclusions. While our observations tend to support Jenkin and Rowley, it must be pointed out that several differences exist between the experimental systems. Thus Jenkin and Rowley induced reticuloendothelial blockade in mice by carbon injection and measured the degree of blockade by the retardation in the clearance of a subsequent injection of carbon. We have used rats and induced acute inhibition by competitive action of two different colloids only one of which was carbon and defined "reticuloendothelial blockade" as the reduction in the rate of carbon disappearance when denatured albumin was administered during the course of carbon clearance.

Although saturation of the phagocytic cell does not appear to us to be the mechanism involved in denatured albumin inhibition of carbon clearance, it is possible that there is a limit to the amount of material that the system can engulf. In fact our experiments were deliberately performed with a relatively low dose of carbon (8 mg/100 gm body weight). It is possible that with higher carbon doses or with multiple carbon injections saturation of phagocytic cells could be achieved; then blockade due to cell saturation might indeed be true. It is entirely possible that this situation does prevail in some cases. In fact it would be reasonable to assume that some form of physiologic limit to the capabilities of the cells to engulf particulate material does exist. However, the presence of a cellular phagocytic rate limit does not exclude a serum factor or factors in phagocytosis, or that a reduction in the circulating level of this factor could lead to apparent "reticuloendothelial blockade." Thus we feel that the concept of reticuloendothelial blockade (at least with respect to certain particles) must now take into account the possibility that the diminished capacity of the system for phagocytosis might arise either from cell saturation or from a limitation in available serum components or both.

The bioassay for the serum factor involved in carbon phagocytosis is not highly sensitive. Barium sulfate extraction, however, allows concentration of the serum factor involved and therefore provides a more sensitive means of search for its presence in whole blood. We now use the barium sulfate method routinely to assay serum for the presence or absence of the factor responsible for preventing denatured albumin from inhibiting carbon clearance. Using this procedure we have now been able to demonstrate that a similar factor (or factors) is present in human, rabbit and hog serum as well as in rat serum. With

this procedure the way is now opened for examination of the amounts of material present in different physiologic and pathologic states as well as for the purication and characterization of the substance(s) involved.

SUMMARY

Reticuloendothelial clearance of carbon particles is inhibited by injection of heat-aggregated albumin. The possibility that this inhibition might involve a serum component has been explored. Evidence that such a serum factor does participate in carbon clearance inhibition by heat-aggregated albumin derives from the fact that prior treatment of the carbon with homologous serum eliminates the blockade; and also, if serum previously absorbed with carbon is mixed with the carbon particles no restoration of "blockade" by albumin occurs. A method for extracting the component from serum using barium sulfate adsorption is described which allows concentration of the factor and sensitive assay for its presence. It is concluded that while large or multiple carbon injections may bring about a saturation of the phagocytic cell, this saturation masks an underlying factor which protects against acute inhibition of carbon phagocytosis by denatured albumin when subsaturating carbon doses are used.

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