THE MECHANISM OF BLOCKADE OF THE RETICULOENDOTHELIAL SYSTEM*

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The rate of clearance of intravenously injected colloidal material is currently believed to be a measure of the phagocytic function of the reticuloendothelial system (RES). Furthermore, it has been assumed that the altered physiologic state observed following blockade of the RES is due to the inability of the RE cells to function as efficiently as before blockade (1, 2). The terms "blockade" and "saturation" of the RES imply that the phagocytic cells are either incapable of ingesting more material or that the rate at which additional particles can be ingested has been markedly retarded (3).

The relationship between blockade of the RES and opsonin levels in blood has not been explored although recent evidence strongly suggests one (4). Plasma proteins and more specifically globulin fractions of plasma have been shown to be necessary for the phagocytosis of colloidal particles (5) and bacteria (6) *in vitro*. More recently the *in vitro* coating of particles by plasma proteins (7) and by a globulin fraction of plasma (8) enhanced their subsequent clearance *in vivo*. Although the nature of these plasma factors or opsonins for particulate material is in doubt it is generally assumed that they are nonspecific.

In the present study the RES was blockaded with various colloidal materials and the effectiveness of the blockade was tested by the subsequent injection of a tracer dose of a similar or dissimilar colloid. It was felt that by using a tracer dose it might be more feasible to completely opsonize the relatively small number of particles, especially with a limited amount of opsonin, and thus provide a more specific test of the relationship of opsonin to blockade of the RES.

In this report it will be demonstrated that blockade of the RES to tracer doses of colloidal material occurs only when the surface properties of the blockading and tracer colloids are similar. Gelatin when used as a colloidal stabilizing agent, confers specificity on the inert particle regardless of the type of particle used. It will be shown that homologous and heterologous plasma

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proteins do not produce a true opsonic effect *in vivo* as contrasted with isologous plasma proteins.

Materials and Methods

Experimental Animals.—Adult albino rats of the Wistar strain were used in all experiments and where indicated a Lewis inbred strain of rats obtained from Microbiological Associates, Inc., Bethesda, were used. The rats were all of the female sex and ranged in weight from 200 to 250 gm. They were fed pellets and water *ad libatum*. Dog plasma was obtained from laboratory animals maintained in this institution.

Particulate Materials.—The non-stabilized, non-radioactive colloids were chromic phosphate (Abbott Laboratories, Chicago), carbon (Excelsior, Binney and Smith, New York), starch (Connaught Laboratories, Toronto), saccharated iron oxide (proferrin, Merck, Sharp & Dohme, Philadelphia), colloidal thorium dioxide (thorotrast, Testagar & Co., Inc., Detroit), and zymosan (Standard Brands Inc., New York). A carbon preparation, C11-1431a (manufactured by Günther Wagner, Hanover, Germany, and obtained commercially from John Henschel & Co., Inc., New York) was also used. Gelatin-stabilized radioactive gold (aurcoloid) and non-stabilized radioactive chromic phosphate were both obtained commercially from Abbott Laboratories.

Stabilizing Agents.—Gelatin (special gelatin P-20, 6 per cent salt-free, Knox Gelatin Co., Inc., Camden, New Jersey), dextran (fraction 478-11-A, Pharmacia, Uppsala, Sweden) and polyvinylpyrrolidone (GA-PVP-118, General Aniline & Film Corp., Easton, Pennsylvania). Gelatin-stabilized carbon was prepared through the courtesy of Dr. George Samuelson of Columbia Carbon Co., Princeton. Gelatin-stabilized colloidal gold was prepared by Abbott Laboratories. Chromic phosphate was stabilized in a 2 per cent gelatin solution by incubation at 37°C on a tissue culture rotor for 48 hours. Carbon was stabilized in 2 per cent dextran and 2 per cent PVP by high speed homogenization in this laboratory. Carbon preparation C11-1431a was suspended in gelatin according to a method previously described (9). Radioactive chromic phosphate was stabilized with gelatin as described for non-radioactive chromic phosphate. All gelatin-stabilized colloids were protected with the same type of special (P-20) gelatin. Prior to injection the radioactive colloids were diluted 100 times in sterile isotonic saline. The volume of the tracer dose varied between 0.05 and 0.1 ml of the diluted radioactive colloid with a maximum activity of 15 microcuries per animal.

Clearance Rates.—The general procedure was similar to that previously described (10). The blockading dose of particulate material was injected *via* the tail vein and 50 minutes later the animal was anesthetized by an intraperitoneal injection of nembutal sodium made up daily in isotonic saline. The jugular veins were exposed and the radioactive tracer dose was injected into one vein approximately 1 hour after the blockading dose. Blood samples (0.12 ml) in heparinized syringes were taken at successive intervals. Aliquots of the blood samples (0.05 ml) in duplicate were measured and placed either in glass vials (radioactive gold) or stainless steel planchets (radioactive chromic phosphate). The radiogold was measured in a well-type scintillation counter and the radiochromic phosphate by a gas-flow thin window GM counter. The time taken for removal of 50 per cent of the circulating radioactivity is designated by T/2.

Preparation of Globulin Fraction.—Cohn's fraction I was prepared from fresh plasma according to Cohn's method as modified by Schmid, Rosa, and MacNair (11). Fraction I precipitate from 20 ml of plasma was dissolved in 10 ml of 0.055 M citrate pH 6.2 ± 0.05 at room temperature. The protein solution was then cooled to 10°C and 20 ml of 12 per cent ethanol in 0.055 M citrate pH 6.2 (*v/v*) cooled to −2°C was added slowly while stirring. After standing for 20 minutes at −2°C, the complex was centrifuged at 3000 RPM for 30 minutes at −2°C. The precipitate was washed with 7 ml of a citrate-saline solution diluted 2 parts

to 1 of water. The citrate-saline solution contained 0.01 M sodium citrate and 0.14 M sodium chloride with the pH adjusted to 6.8. The precipitate was centrifuged as before and dissolved in 1 ml of citrate-saline. The clottable protein was removed by conversion to fibrin with thrombin. Commercial bovine thrombin (Parke, Davis & Co.) was dissolved in a 50 per cent glycerol-saline solution in a concentration of 200 NIH units per ml and stored at −20°C until used. Thrombin (0.4 NIH units per ml of original plasma volume) was added to the protein solution and incubated at 37°C for 1 hour. The clot was centrifuged at 24,000 *g* for 20 minutes at 10°C and the supernatant globulin fraction removed. The colloidal material was added to the globulin solution and carefully mixed by a syringe. Calcium and magnesium

TABLE I

Blockade of the RES by Gelatin-Stabilized Colloids Tested 1 Hour after Blockade with Gelatin-Stabilized Radioactive Particles and Non-Stabilized Radioactive Particles

No. of Animals	Blockading agent stabilized in 2 per cent solution of gelatin	Blockading dose, *mg per 100 gm body weight*	Tracer injection	T/2 (min.) tracer dose	Effect
10			Gelatin-stabilized Au^{198}	0.92 ± 0.07*	
5			Gelatin-stabilized radio-$CrPO_4$	0.94 ± 0.09	
6			Radio-$CrPO_4$ in saline	0.61 ± 0.06	
10	Gelatin‡	10	Gelatin-stabilized Au^{198}	7.0 ± 0.11	Blockade
4	Carbon	8	"	16.4 ± 0.43	Blockade
8	Gold	4	"	19.2 ± 0.90	Blockade
4	Chromic phosphate	5	"	13.8 ± 0.45	Blockade
5	Gelatin‡	10	Gelatin-stabilized radio-$CrPO_4$	8.5 ± 0.24	Blockade
5	Carbon	8	"	17.1 ± 0.58	Blockade
4	Gold	4	"	15.3 ± 0.33	Blockade
4	Chromic phosphate	5	"	14.6 ± 0.40	Blockade
5	Gelatin‡	10	Radio-$CrPO_4$ in saline	0.68 ± 0.07	No blockade
4	Carbon	8	"	0.71 ± 0.09	No blockade
6	Gold	4	"	0.65 ± 0.10	No blockade
5	Chromic phosphate	5	"	0.60 ± 0.08	No blockade

* SE of the mean.

‡ Gelatin injected alone as a 5 per cent solution.

chloride solutions were added to make a final concentration of 0.02 M Ca^{++} and 0.005 M Mg^{++}. The suspension was mixed again, the pH adjusted to 7.0 and incubated at 37°C for 30 minutes. The globulin fraction prepared from 5 and 20 ml of plasma was used to opsonize tracer and blockading doses respectively of the particulate materials.

Results

Blockade Studies.—In previous studies (10) as well as the investigations of others (12) the half-time clearance rates of intravenously injected tracer amounts of colloid has been approximately 1 minute or less. A half-time clearance rate of 4 minutes or more has been arbitrarily chosen in this study as evidence that a blockade to the clearance of the tracer dose of colloid has occurred. Such a half-time is well outside the normal variation in clearance rates in non-treated rats. Blockade of the tracer colloid occurred when both the

blockading and tracer colloids were stabilized with the same material; *i.e.*, gelatin (Table I). When the type of stabilized particle differed in the two injections the result was the same as when similar stabilized particles were injected. However, when the blockading colloid was stabilized with gelatin and the tracer colloid was non-stabilized no blockade of the tracer dose occurred regardless of the type of particle used. The injection of gelatin alone as the

TABLE II

Blockade of the RES by Various Colloids Tested 1 Hour after Blockade with Gelatin-Stabilized and Non-Stabilized Radioactive Chromic Phosphate

No. of animals	Blockading agent	Blockading dose, per 100 gm body weight	Tracer injection	T/2 (min) tracer dose	Effect
5	Carbon*	16 mg	Radio-$CrPO_4$ in saline	0.66 ± 0.09‡	No blockade
6	Carbon*	16 "	Gelatin-stabilized radio-$CrPO_4$	0.88 ± 0.12	No blockade
6	Gelatin-stabilized carbon*	16 "	Gelatin-stabilized radio-$CrPO_4$	19.6 ± 0.58	Blockade
5	$CrPO_4$ in saline	4 "	Radio-$CrPO_4$ in saline	8.4 ± 0.24	Blockade
4	Dextran-stabilized carbon	8 "	Radio-$CrPO_4$ in saline	0.61 ± 0.06	No blockade
4	Dextran-stabilized carbon	8 "	Gelatin-stabilized radio-$CrPO_4$	0.85 ± 0.08	No blockade
5	PVP - stabilized carbon	8 "	Radio-$CrPO_4$ in saline	0.60 ± 0.06	No blockade
4	PVP - stabilized carbon	8 "	Gelatin-stabilized radio-$CrPO_4$	0.91 ± 0.07	No blockade
6	Starch	10 "	Radio-$CrPO_4$ in saline	0.58 ± 0.05	No blockade
5	Starch	10 "	Gelatin-stabilized radio-$CrPO_4$	0.94 ± 0.08	No blockade
7	Proferrin	6.7mg	Radio-$CrPO_4$ in saline	0.62 ± 0.06	No blockade
6	Proferrin	6.7 "	Gelatin-stabilized radio-$CrPO_4$	0.88 ± 0.10	No blockade
7	Thorotrast	0.1 ml	Radio-$CrPO_4$ in saline	0.61 ± 0.04	No blockade
5	Thorotrast	0.1 "	Gelatin-stabilized radio-$CrPO_4$	0.95 ± 0.09	No blockade
6	Zymosan	4 mg	Radio-$CrPO_4$ in saline	0.59 ± 0.07	No blockade
6	Zymosan	4 "	Gelatin-stabilized radio-$CrPO_4$	0.88 ± 0.09	No blockade

* Carbon preparation C11-1431a.

‡ SE of the mean.

blockading dose resulted in blockade only when the tracer dose was stabilized by gelatin. A similar relationship was demonstrated for non-stabilized particles; *e.g.*, chromic phosphate (Table II). To further explore this phenomenon various other colloids were injected as the blockading material. Without exception the same general rule was observed. These studies suggested therefore that blockade to a tracer dose of colloid occurred only when the surface properties of both blockading and tracer particles were identical. This interrelationship between specific surface properties of particles and blockade of the RES suggested the possible deficiency of specific plasma factors or opsonins which have been shown to enhance the rate of phagocytosis *in vitro* as well as *in vivo*.

TABLE III

The Effect of Homologous and Isologous Opsonins on Blockade of the RES by a Gelatin-Stabilized Colloid

No. of animals	Experimental rat strain	Source of plasma opsonin	Blockading agent	Blockading dose mg per 100 gm body weight	Tracer injection	T/2 (min) tracer dose	Effect
8	Wistar		Gelatin-stabilized gold colloid	4	Gelatin-stabilized Au^{198}	19.2 ± 0.90*	Blockade
10	Wistar	Wistar rat	Non-opsonized gelatin-stabilized gold colloid	4	Opsonized gelatin-stabilized Au^{198}	0.95 ± 0.11	No blockade
8	Wistar	Wistar rat	Opsonized gelatin-stabilized gold colloid	4	Opsonized gelatin-stabilized Au^{198}	23.6 ± 1.6	Blockade
7	Wistar	Dog	Non-opsonized gelatin-stabilized gold colloid	4	Opsonized gelatin-stabilized Au^{198}	1.1 ± 0.09	No blockade
8	Wistar	Dog	Opsonized gelatin-stabilized gold colloid	4	Opsonized gelatin-stabilized Au^{198}	27.2 ± 1.4	Blockade
10	Lewis	Lewis rat	Non-opsonized gelatin-stabilized gold colloid	4	Opsonized gelatin-stabilized Au^{198}	0.91 ± 0.08	No blockade
7	Lewis	Lewis rat	Opsonized gelatin-stabilized gold colloid	4	Opsonized gelatin-stabilized Au^{198}	0.98 ± 0.06	No blockade
6	Lewis	Lewis rat	Opsonized gelatin-stabilized chromic phosphate	5	Opsonized gelatin-stabilized radio-$CrPO_4$	0.95 ± 0.08	No blockade

* SE of the mean.

The Effect of Opsonins.—If opsonin was a non-specific protein it would be difficult to interpret the above findings. However, if the type of protein that coated an injected particle was specifically determined by the surface properties of that particle then it should be possible to supply the specific opsonin *in vitro* and thus prevent blockade of the tracer colloid. When opsonin prepared from Wistar strain rats was used to coat the tracer colloid there was no evidence of blockade (Table III). To test whether this represented a true opsonin effect or merely the presentation of tracer particles with different surface properties, the blockading colloid was similarly coated *in vitro* with the same type of opsonin. Blockade to the opsonized tracer dose now occurred. The experiment was repeated using opsonin prepared from dog plasma with the same result. These findings were consistent with the results shown in Tables I and II, and further suggested that opsonins may be not only specific for the injected particle but are also specific for the individual. As a further experiment the globulin fraction was prepared from plasma obtained from an inbred strain of female rat. Opsonization of both blockading and tracer doses of colloid with isologous globulin did not result in blockade of the tracer colloid.

DISCUSSION

In studies of clearance rates of stabilized colloids from the circulation more emphasis is often given to the type of particle used rather than to the stabilizing agent. It has been previously reported that increasing concentrations of the stabilizing agent, gelatin, resulted in decreasing rates of clearance of a constant number of gold particles (10). A possible mechanism for this effect of gelatin may be indicated from the results shown in Table I. The blockading effect of a gelatin-coated particle when subsequently tested with a similarly stabilized particle is dependent on the surface properties of the particle; *i.e.*, gelatin and not on the nature of the particle itself. Thus by increasing the concentration of gelatin in a gelatin-stabilized colloid, the decreasing rates of clearance might be explained by the increased number of gelatin particles presented to the blood plasma. There is only a difference in the degree of blockade when gelatin alone is used as the blockading agent and it has been demonstrated that gelatin was phagocytized by Kupffer cells in locations of the liver lobule that were identical with a gelatin-stabilized colloid (13). Since gelatin forms a protective coating around colloidal particles a greater surface area of gelatin would be presented to the blood plasma than the injection of a 5 per cent gelatin solution. Moreover the amount of opsonin that would be required to coat a gelatin-stabilized colloid would be many times that required to coat masses of gelatin even if the total quantity of gelatin injected in both instances are identical. Some support for this thesis is suggested by Zinnser's calculation (14) that the amount of immune antibody absorbed from serum was dependent on the surface area of the antigen presented.

It has been established previously that the rate of clearance of particulate materials is dependent on the number of particles injected (15). Decreasing rates of clearance of successive injections of particulate materials has been explained by the saturation effect on the RES (15). However, if opsonins were limited in quantity then an equally satisfactory explanation could be given for the dose-clearance relationship. The demonstration that blockade of the RES was produced by the successive injection of particles with similar surface properties might imply that specific opsonin is involved in the phagocytosis of colloidal material *in vivo*. Evidence from the literature for or against such a belief is meager. Dobson reported that there was no blockade to a tracer dose of yttrium following a blockading injection of colloidal chromium phosphate (12). Plasma proteins either recognize differences in the surface properties of inert particles or the surface properties of particles differ in their affinity for plasma proteins. Phagocytosis of amber particles and cream droplets was retarded in the frog and was associated with a delay in the acquisition of a coating material from the plasma while opsonization and phagocytosis of India ink were both rapid (16). When carbon and quartz particles were presented to leucocytes *in vitro*, the higher ratio of carbon particles phagocytized was attributed to the more rapid opsonization of the carbon (17).

Phagocytosis of particles *in vivo* may be a more complex mechanism as compared to phagocytosis of the same particle *in vitro*. Gold colloid suspended in saline was not phagocytized in the perfused liver but if stabilized by gelatin phagocytosis was now as efficient as when coated with serum protein (18). However, blockade is readily produced by the injection of gelatin-stabilized colloids *in vivo*. The clearance of particulate materials from the circulation resembles the kinetics of bacterial removal from the blood and serum factors appear to play a prominent role in both. The specificity of natural opsonins has been demonstrated in studies of the phagocytosis of bacteria by leucocytes *in vitro* (19). The natural resistance of some species to infection by a specific organism was associated with the presence of a specific natural opsonin in their sera (20, 21). Moreover, phagocytosis of bacteria by leucocytes obtained from the susceptible species was highly efficient when opsonized with normal serum from the resistant species. In contrast to the *in vitro* studies, blood clearance rates were enhanced in the present study when both blockading and tracer doses of the colloid were coated with isologous globulins but not enhanced when opsonized with homologous or heterologous globulins, again suggesting differences between *in vivo* and *in vitro* phagocytosis. It has been emphasized (22) that there is no proof of causal relationship between phagocytic activity and resistance to infection and that rapidity of removal of bacteria from the blood may be but one factor in determining resistance to circulating organisms.

The opsonization of particles with heterologous and homologous plasma *in vitro* enhances their subsequent clearance from the circulation but this is

probably only indirect evidence for opsonins as a factor in clearance rates. The *in vitro* coating of chromic phosphate with gelatin when injected into an animal previously injected with a blockading dose of non-stabilized chromic phosphate shows a similar opsonic effect of gelatin. It is suggested that the *in vitro* coating of particles and bacteria by heterologous and homologous plasma proteins is another example of the same phenomenon. Moreover, when heterologous and homologous plasma was injected into rats to provide excess opsonin prior to the injection of colloidal particles, there was a marked decrease in the rate of clearance of the particles (10). It would appear from the results presented in this study that the heterologous and homologous globulin used to coat the colloid *in vitro* is treated as any other foreign protein in the host by being coated in turn by autologous opsonin.

SUMMARY

Rats were injected intravenously with various particulate materials with and without the addition of various stabilizing agents. One hour after the injection of the blockading colloid, a tracer dose of similar or different colloidal material was injected. A half-time clearance rate of the tracer dose greater than 4 minutes was taken as evidence of blockade of the RES.

Blockade of the tracer dose occurred when the surface properties of the particles in both blockading and tracer doses were identical. Different particles stabilized by the same agent behaved as identical particles and identical particles stabilized by different agents behaved as different particles.

The opsonization of a tracer dose of gelatin-stabilized colloid *in vitro* by a specific globulin fraction obtained from heterologous and homologous plasma prevented its blockade, while opsonization of both blockading and tracer doses with the same proteins resulted in blockade. However, when both blockading and tracer doses were opsonized with isologous globulins, blockade did not occur.

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