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Research Article

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Influence of Antigen on Immune Complex Behavior in Mice

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ABSTRACT To explore the possibility that the behavior of immune complexes can, under some circumstances, be directed by the antigen, we have studied the behavior of complexes of identical size made with the glycoproteins, orosomucoid (OR), and ceruloplasmin; or with their desialylated derivatives, asialo-orosomucoid (ASOR) and asialo-ceruloplasmin. Such desialylated proteins are rapidly removed from the circulation by a hepatic cell receptor for galactose, the sugar exposed upon removal of sialic acid.

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INTRODUCTION

Immunoglobulins are a set of heterogenous molecules which, upon reacting with antigen, can transmit common effector messages to complement, to macrophages, to lymphocytes, and to other cells. Antigen initiates this process by binding to the Fab regions of immunoglobulin molecules, which results in a multimolecular complex with grouped Fc pieces. Although the Fc pieces have no specificity for the antigen and appear to remain largely or entirely unaltered after antigen binding, all further reactions of the complex have been considered to involve interaction of the grouped Fc pieces with cellular receptors or serum

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proteins (1). In this view, antigens merely aggregate Fc pieces, thereby allowing effector systems to distinguish the aggregated Fc pieces of the immune complex from the monomer Fc pieces of uncomplexed immunoglobulins. Since the effector systems can also recognize Fc pieces aggregated without antigen, e.g., by heat or chemical aggregation (2), such a view seems adequately to explain the normal or ideal operation of the humoral immune system.

There has been considerable study of those properties of immune complexes and of the effector systems that might allow complexes to circulate abnormally and deposit at abnormal sites (3–5). The size of complexes certainly influences the length of their survival in the circulation (6), their ability to fix complement (7), and the affinity of their binding to reticuloendothelial Fc receptors (8). The class or subclass of antibody also affects those properties (9). A failure of Fc or complement receptor function might lead to prolonged survival of complexes, as might a depletion of certain complement components.

But the size, immunoglobulin class, and complement-fixing ability of complexes do not by themselves seem able to account for the great variety of tissue localizations and subsequent reactions that have been ascribed to immune complexes. It is not clear why, for example, some complexes deposit in the glomerular basement membrane and others do not. It is possible that antigen may deposit first in a tissue and that antibody follows (10). The interaction of DNA within glomerular or epidermal basement membranes, for example, may direct antibody to those sites (11). In addition, the charge-selective properties of the glomerulus influence which molecules pass freely and which are retained (12). This anionic membrane may also be important in regulating the pattern of immune complex deposition within the glomeruli (13). When antibodies are present in the blood, however, antigen may not circulate freely. Since immunoglobulins have only a narrow range of charge variation, the fate of antigen-antibody complexes may depend not only on the state of aggregation of the Fc pieces but also on direct interactions of the antigen with the tissues.

It has recently been appreciated that many cells have receptors for glycoproteins which are specific to the terminal nonreducing sugar of the carbohydrate side chain (14). These receptors have been characterized on hepatocytes, mononuclear phagocytes, and fibroblasts. Mammalian hepatocytes have a receptor that recognizes terminal galactose, whereas macrophages recognize either mannose or *N*-acetyl-glucosamine. If the antigen in an immune complex is a glycoprotein capable of binding to specific sugar receptors, the complex might behave in a pattern dependent more on the antigen than the antibody.

To determine whether or not the antigen in an immune complex can play a critical role in determining the fate of that complex, we have studied the way in which pairs of closely related antigens, which form immune complexes equally well with the same purified antibodies (15), influence the clearance and tissue localization of the immune complexes they form. The only difference in primary structure between the two antigens in each pair is that the desialylated derivatives had galactose rather than sialic acid as the terminal sugar on the carbohydrate side chains. The desialylated glycoproteins readily bind to galactose receptors on hepatocytes (16), thereby raising the possibility that immune complexes containing them might bind to receptors via either the Fc piece on immunoglobulin or the galactose exposed on antigen. In experiments with the antigen pairs orosomucoid (OR)/asialo-orosomucoid (ASOR)[1] and ceruloplasmin/asialo-ceruloplasmin, we have shown that complexes formed from the same purified antibodies and of the same size are handled in dramatically different ways when the antigens differ only in the presence or absence of terminal sialic acid residues on the carbohydrates side chains of the antigen.

METHODS

Antigens and protein inhibitors. Human OR was generously provided by Dr. M. Wickerhauser of the American Red Cross Research Center, Bethesda, Md. Fetuin was purchased from Grand Island Biological Co. (Grand Island, N. Y.), and human ceruloplasmin type III was purchased from Sigma Chemical Co. (St. Louis, Mo). OR and fetuin were desialylated using *Diplococcus pneumoniae* neuraminidase as previously described (17). To prepare asialo-ceruloplasmin, a solution of ceruloplasmin at 50 mg/ml was dialyzed against 0.1 M sodium acetate buffer, pH 6.5. Purified neuraminidase was added in excess and the mixture was kept at 37°C overnight, and then filtered twice through a 1.9 × 95-cm column of Sephadex G-150 in 0.16 M NaCl:0.2 M sodium borate, pH 8.0 (borate-buffered saline) to remove the enzyme and contaminating larger proteins found in the purchased material. The ratio of absorbance at 610 nm to that at 280 nm was 0.039 in the final material. Human gamma globulin Cohn fraction II (Miles Laboratories, Inc., Elkhart, Ind.) was heat aggregated as described by Dickler and Kunkel (2). We prepared galactose-Ficoll by making aminoethylcarboxymethyl Ficoll 70 as described by Inman (18), and derivitizing it with galactose using cyanomethyl-1-thio-β-D-galactopyranoside (Sigma Chemical Co.), as described by Lee (19). (Full publication of the method is in preparation.)

Antibody preparation and isolation. Goat serum containing antibodies raised against ASOR was a kind gift of Dr. J. Robbins, Bureau of Biologics, National Institutes of Health, Bethesda, Md. To obtain rabbit antibodies outbred New Zealand white rabbits were injected at multiple subcutaneous and intramuscular sites with 3 mg OR or ceruloplasmin emulsified in complete Freund's adjuvant and, 3 wk later, with the same amount emulsified in incomplete Freund's adjuvant. 6 wk later, when the sera contained 0.7–0.9 mg/ml of anti-OR antibodies or 3–4 mg/ml of anticeruloplasmin antibodies, as

[1] *Abbreviations used in this paper*: ASOR, asialo-orosomucoid; HAG, heat-aggregated gamma globulin; OR, orosomucoid.

determined by precipitin test, animals were exsanguinated. Although all the experiments reported here were carried out on sera obtained from single rabbits, we have found similar results with sera from other rabbits. The rabbit sera were heated to 56°C for 30 min before antibody purification. The antibodies were purified by passing the serum over OR or ceruloplasmin affinity columns, prepared as described (20). For anti-OR antibodies, 15 ml of anti-OR antiserum was applied to a 0.7 × 6.5-cm OR-Sepharose 4B column equilibrated with 0.02 M Tris, pH 7.8, 0.15 M NaCl. After extensive washing with the same buffer, antibodies were eluted with 0.15 M NaCl, 0.20 M acetic acid; fractions of 1 ml were collected into tubes containing 0.25 ml of 1 M sodium acetate, pH 5.3. For anticeruloplasmin antibodies a ceruloplasmin affinity column was used, elution was with 1 M acetic acid, and the pooled protein-containing fractions were neutralized with dilute NaOH. The eluted antibodies were dialyzed against borate-buffered saline.

Mouse IgG antidinitrophenyl model immune complexes were prepared with the bis-affinity labeling reagent, bis-2, 4-dinitrophenyl pimelic ester, as described (21).

Radioiodination of antibody and antigen. Antibodies were radiolabeled with carrier-free $Na^{125}I$ (New England Nuclear, Boston, Mass) with a modified iodine monochloride method at 1 mCi/mg protein, as previously described (22). Antigens were radiolabeled with carrier-free $Na^{131}I$ with either the modified iodine monochloride method at 1 mCi/mg or chloramine-T (23) at 5 mCi/mg protein.

Preparation of immune complexes. Soluble immune complexes were prepared by adding antigen to a solution of purified antibodies and separating unreacted antibodies and antigens from complexes by density gradient centrifugation. In a series of preliminary experiments, trial sucrose gradients of antigen-antibody mixtures with different ratios (all in the region of antigen excess as determined by a precipitin test) were done to find the ratio yielding the largest amount of heavy soluble complexes. For the goat antibody this was achieved by adding eightfold more antigen than equivalence. For the rabbit antibody, 1.75-fold more antigen than equivalence was optimal. In all cases the native and desialylated antigens behaved identically with respect to the formation of soluble complexes.

To prepare complexes for injection, 20 μg of ^{125}I-antibody was combined with either 12.4 μg of nonradioactive or ^{131}I-antigen (goat antibodies) or 3.1 μg of antigen (rabbit antibodies) in a final volume of 200 μl. After incubation at room temperature for 30–60 min, the mixture was layered on top of a 4.8-ml 10–30% linear sucrose gradient in 0.15 M NaCl-0.01 M sodium phosphate, pH 7.4. Gradients were placed in a Beckman SW50.1 rotor and centrifuged at 40,000 rpm for 13–14 h in a Beckman model 65LB centrifuge at 0°C (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The bottom of the tube was punctured and fractions containing three drops each were obtained. Generally there were 28 fractions per gradient. Fig. 1 shows a typical gradient pattern for the goat and rabbit antibodies. Identical gradient patterns were obtained with OR and ASOR. Similar sucrose gradients of antibody alone or in complexes with antibody excess showed that there was no self-aggregation of the IgG molecules.

Of the goat complexes the first 11 fractions were pooled and injected without further handling. These fractions contained complexes existing primarily of trimers and tetramers. The fractions from gradients containing rabbit complexes were arbitrarily divided into three regions, designated heavy, medium, and light (Fig. 1), which were composed of complexes of the following size, respectively, Ab6-7:Ag3-4, Ab4-6:Ag2-3, and Ab3-4:Ag1-2. These values were derived from the antibody:antigen ratio of 2, observed when both components were radioactively labeled and from molecular weight markers on a simultaneously run gradient. The respective size fractions were pooled and usually diluted 1:1 before injection. Complexes were used only on the day of preparation to avoid further aggregation, which we had observed when complexes were allowed to stand for several days.

Since immune complexes were prepared at a point where not all antibodies were combined with antigen, it was necessary to establish that both OR and ASOR were interacting with the same antibodies. To do this, we tested the ability of each to inhibit precipitation by the other. To a constant quantity of purified antibody solution we added enough OR or an identical quantity of ASOR to yield a precipitate after overnight incubation. Simultaneously, either a 10-fold excess of the homologous or heterologous antigen, or buffer was added. The degree of inhibition of precipitation was measured by comparing precipitates in the presence of buffer and in the presence of excess antigen. The ability of the two antigens to inhibit precipitation was independent of the precipitating antigen used. This established that both antigens reacted equally well with the same antibody molecules, not merely with some of the molecules in the purified preparation. Furthermore, analogous experiments, in which a 10-fold excess of the same or the other antigen was added to mixtures before density gradient centrifugation, showed that a reduction in the size of complexes from heavy oligomers to dimer and monomer could be accomplished equally well with an excess of either antigen in the presence of the other. To prove that both ASOR and OR could react with the same goat antibody molecules, an affinity column of the purified goat antibodies was made. Unlabeled ASOR and OR passed over the column were able indistinguishably to inhibit the binding of radiolabeled ASOR.

Complexes with either intact ceruloplasmin or asialo-ceruloplasmin were made by mixing radioiodinated antibody at twofold antigen excess of equivalence (as determined of a quantitative precipitin test) with either ceruloplasmin or asialo-ceruloplasmin. After a 30-min incubation at room temperature, the complexes were separated into heavy, medium, and light weight fractions over a sucrose density gradient, as described above for the rabbit anti-OR complexes. The complexes were used on the day of preparation and diluted 1:1 before intravenous injection.

In vivo blood clearances. 1–3 μg of the radiolabeled antibody-antigen complex or antigen alone in a volume of no more than 0.3 ml was injected via the tail vein into 20-g C3H female mice. Starting at 1 min, blood samples were obtained over 30–60 min from the retroorbital sinus using a 25-μl calibrated micropipette (Clay-Adams Inc., Parsippany, N. Y.). The blood samples were then expelled into 1.5-ml plastic microfuge tubes and assayed for radioactivity with a Beckman Gamma 8000 spectrophotometer (Beckman Instruments). The clearance curves for the complexes are presented as percentage of ^{125}I radioactivity (antibody-associated radioactivity) remaining in the blood, the 1-min sample serving as zero time and 100%. For antigen alone, the trichloroacetic acid-precipitable radioactivity was used to generate the clearance curve.

In vivo uptake of immune complexes by the liver. A known amount of the radiolabeled immune complex was injected as described above. Either 1 h after injection (goat-OR, goat-ASOR, and rabbit-OR complexes) or 30 min after injection (rabbit-ASOR complexes), the animal was killed by cervical dislocation, and the amount of the radioactivity within the liver determined. The contaminating blood volume of the liver of a representative group of animals given complexes in various sucrose concentrations was determined

using ^{51}Cr-labeled autologous erythrocytes as a marker of blood volume (21). The radioactivity due to blood within the liver (always < 10%) was substracted from the total radioactivity in the liver to give a corrected value for the amount of immune complexes bound within the liver. The amount of ^{125}I within the liver is expressed as a percentage of the total amount injected. Less than 1% of the injected radioactivity was found within the spleen and kidneys.

Inhibition studies. 15 min before injection of a tracer dose of radiolabeled immune complexes, 2–3 mg of the inhibitor was injected via the tail vein. After the injection of the immune complexes, serial blood samples were obtained as described. After the last sample the animal was killed, and the amount of radioactivity within the liver measured as described above. Since the inhibitors with galactose-receptor blocking activity have different numbers of terminal galactose residues capable of binding to the receptor, the same degree of inhibition of clearance or hepatic uptake per milligram of inhibitor injected should not be expected (16). The data are presented as a percentage of injected radioactivity within the liver and as the percentage of suppression of uptake induced by the inhibitor.

Autoradiography. OR and ASOR complexes made with rabbit antibodies were made as described above and were injected into the animal without prior separation on the sucrose density gradient. This was done to prevent the vacuolization of hepatocytes that occurs after the injection of sucrose. Since a negligible amount of uncomplexed antibody is found in the liver after intravenous injection (21), the autoradiographs reflect the uptake of complexes. Enough material was injected to allow for 10^5 cpm to be taken up into the liver. The animal was killed and the liver placed in 10% formalin. 5-μm sections were prepared and deparafinized. The deparafinized sections were dipped into NTB-2 Nuclear Tract Emulsion (Eastman Kodak Co., Rochester, N. Y.) and allowed to remain for 10 d at 4°C. The slides were then developed with D19 (Kodak), fixed with Rapid fixer (Kodak), and stained lightly with hematoxalin and eosin.

Liver cell separation studies. A modification of the method of Steer et al. (24) was used for the isolation and purification of mouse hepatocytes and nonparenchymal cells. Mice were anesthetized with 20 mg sodium pentobarbital i.p. After laparotomy the portal vein was isolated and cannulated with a 22-gauge Angiocath (Deseret Pharmaceuticals, Sandy, Utah), which was passed to the porta hepatis. The inferior vena cava was then cannulated with a 22-gauge Angiocath. The liver was flushed via the portal vein with 2 ml of warmed, oxygenated Krebs-Henseleit buffer without calcium, pH 7.4, and then connected to a perfusion apparatus as described by Krebs et al. (25). The gas phase was maintained at 95% O_2:5% CO_2. The perfusate, which contained warmed Krebs-Henseleit buffer without calcium, pH 7.4 and collagenase IV (Sigma Chemical Co.) at a final concentration of 0.03% (wt/vol), was maintained at a flow rate of 3 ml/min.

After 10 min of perfusion the liver was excised, cut into small pieces, and placed in 40 ml of Krebs-Henseleit buffer, pH 7.4, containing 2.5 mM calcium. After 2 min the liver was pressed through a 40-gauge mesh filter with the barrel of a plastic syringe (Monoject, Sherwood, Medical Industries, Inc. Deland, Fla.) and then refiltered four times. All further operations were carried out at 4°C. The cell suspension was sedimented at unit gravity for 10 min. The resulting sediment, which contained primarily hepatocytes and cellular debris, was resuspended and washed three times in Krebs-Henseleit buffer containing 2.5% bovine serum albumin (wt/vol) after centrifugation at 50 *g* for 3 min, and was then resuspended in 20 ml of fresh Waymouth's medium 752/L containing 17.5% (vol/vol) heat-inactivated newborn calf serum. The supernate, which contained primarily nonparenchymal cells, was layered gently over 16% (wt/vol) Metrizamide (Sigma Chemical Co.), pH 7.6, containing 0.24% Hepes, 0.05% KCl, and 0.018% CaCl:2H_2O and centrifuged at 3,000 *g* for 45 min. The purified nonparenchymal cells, containing primarily Kupffer and endothelial cells, were recovered from the Metrizamide supernatant interface. These cells were washed three times in Krebs-Henseleit buffer, pH 7.4, and then resuspended in Earle's minimal essential media and 17.5% newborn calf serum. Cells were counted using a hemocytometer.

Total recovery ranged from 2 to 5 × 10^7 hepatocytes (20–50%) and 0.2 to 1 × 10^7 nonparenchymal cells (5–25%) per liver, with a viability as assessed by trypan blue exclusion of 40–80% for hepatocytes and 98–99% for nonparenchymal cells. Contamination of hepatocytes in the nonparenchymal cell preparation and nonparenchymal cells in the hepatocyte preparation, as determined by counting 200 cells from each preparation, was <1%. The radioactivity in 1 × 10^6 cells from each preparation (hepatocytes and nonparenchymal cells) was determined with a Beckman 8000 gamma scintillation counter (Beckman Instruments, Inc.). Since hepatocytes are ~27-fold larger in volume than nonparenchymal cells, the data were calculated from the radioactivity per volume of cells, which correlates well with presenting the data as radioactivity per milligram of cell protein (25, 26).

RESULTS

In vivo clearance and hepatic uptake of antigen and of goat immune complexes. ASOR, when injected alone intravenously, has a $t_{1/2}$ in the circulation of <3 min, in contrast to OR which has a $t_{1/2}$ of >1 h (Fig. 2). After 15 min there is a slight increase in trichloroacetic acid precipitable radioactivity, the cause of which we have not further investigated. Galactose receptors on hepatocytes recognize ASOR and are responsible for its rapid clearance in vivo (16). To determine whether ASOR when complexed to an antibody could influence the behavior of that complex, we prepared immune complexes from radiolabeled goat anti-OR antibodies using unlabeled ASOR or OR. Identical-sized complexes were formed with either antigen at the same

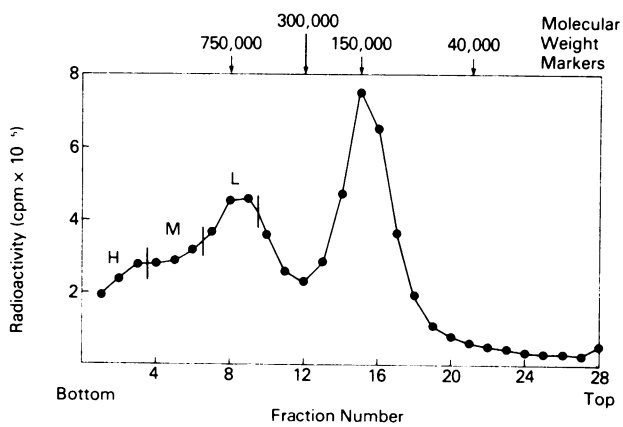

FIGURE 1 Fractionation pattern of soluble immune complexes separated over a 10–30% linear sucrose gradient. H, heavy complexes; M, medium complexes; L, light complexes. (See text for details.)

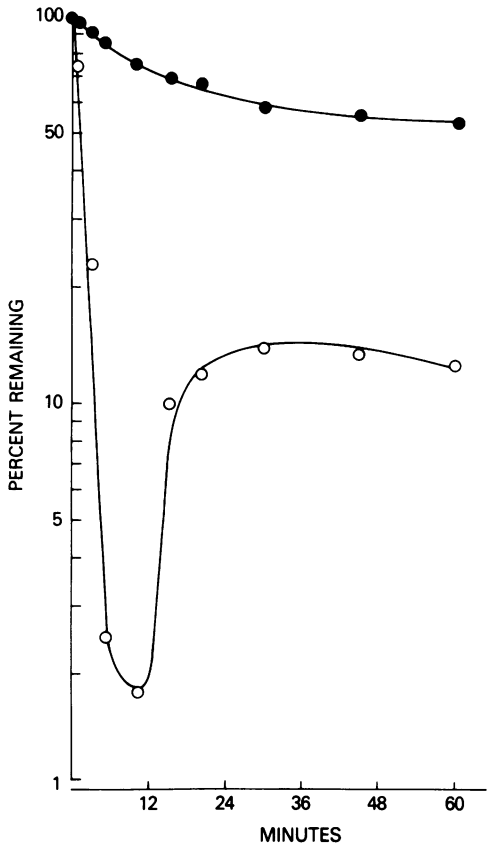

FIGURE 2 The blood clearance of ASOR and OR, alone, in C3H mice. Each point is the mean of two animals and represents trichloroacetic acid precipitable radioactivity. ●, OR; ○, ASOR.

concentration. The injected complexes were composed of three to four antibody molecules and two to three antigen molecules. ASOR complexes were cleared much more rapidly than OR complexes (Fig. 3). 1 h after injection 10% of the OR complexes were within the liver, whereas 37% of the ASOR complexes were in the liver at that time (Fig. 3). Because complexing of OR with goat antibody did not markedly alter its rate of clearance, whereas, once complexed with antibody, ASOR was removed from the circulation somewhat more slowly (Fig. 3), the differences observed in the clearance curves for OR and ASOR immune complexes (Fig. 3) therefore predominantly resulted from the differences in the structure between the two antigens; that is, the exposure of a penultimate galactose on ASOR. (Although the radiolabel followed in Fig. 3 was within the antibody, the clearance curves were similar when the radiolabel followed was within the antigen.) Because ASOR complexed with antibody remained circulating longer than in an uncomplexed state, this might enhance the antigenicity of ASOR, thereby possibly enabling more immune complexes to form.

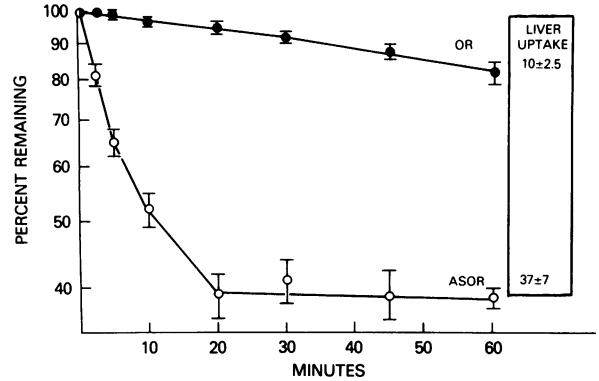

FIGURE 3 The effect of antigen on the blood clearance of goat anti-ASOR soluble immune complexes. ●, OR complexes; ○, ASOR complexes. Cross bars represent mean±SEM. There were five animals in each group.

A blocking dose of uncomplexed ASOR (2 mg) markedly suppressed the in vivo clearance and hepatic uptake of the ASOR complex (Figs. 3 and 4; Table I). In contrast, the rapid clearance and hepatic uptake of the ASOR complex was essentially unaffected by a prior injection of human heat-aggregated gamma globulin (HAG) in a dose known to block 60–75% of the available Fc receptors in the liver (5) (Fig. 4, Table I). Two mechanisms act together to prolong the half-life induced by the blocking dose of ASOR: first, the ASOR would block galactose receptors on hepatocytes; and second, it may reduce the size of the complexes by providing antigen excess within the circulation. A prior injection of asialo-fetuin, however, an unrelated glycoprotein with exposed galactose residues but not recognized by the antibodies, also resulted in moderate inhibition of the clearance of the ASOR complexes. This supports an interaction of ASOR complexes with liver galactose receptors. Large doses of OR or intact

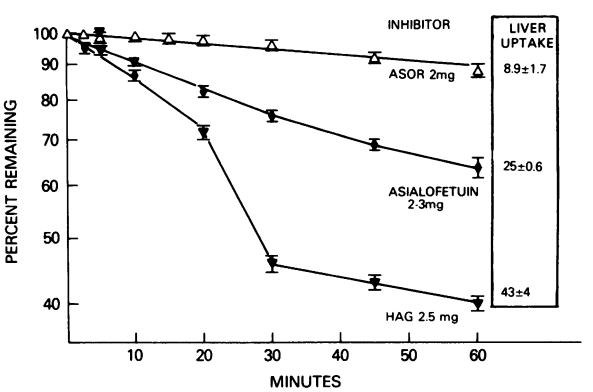

FIGURE 4 The effect of a prior injection of specific inhibitors on the blood clearance of goat ASOR soluble complexes. Inhibitors (dose): △, ASOR (2 mg) (n = 3); ◆, asialofetuin (2–3 mg) (n = 2); ▼, HAG (2.5 mg) (n = 4). Cross bars represent mean±SEM.

TABLE I

Effect of Antigen on the Blood Clearance of Similarly Sized Soluble Immune Complexes Made with Goat Antibody

Inhibitor*	Antigen			
	ASOR		OR	
	n	$t_{1/2}$ (min)‡	n	$t_{1/2}$ (min)‡
None	5	15	5	300
HAG	4	28	ND	
ASOR	3	300	ND	
Asialofetuin	2	98	ND	
OR	4	20	ND	
Fetuin	2	30	ND	

* 2–3 mg of the inhibitor was injected 15 min before the ^{125}I immune complex.

‡ $t_{1/2}$ represents the point at which 50% of the injected complexes has been removed from the calculation.

ND, not determined.

fetuin (3 mg) had a negligible effect on the in vivo survival of the ASOR complex (Table I).

In vivo clearance and hepatic uptake of rabbit complexes. Because goat OR complexes were cleared poorly after intravenous injection into mice, probably because mouse Fc receptors do not recognize the goat Fc piece (27), we studied the behavior of complexes prepared with purified rabbit anti-OR. Mouse liver reticuloendothelial cells have been shown to recognize rabbit immunoglobulin in immune complexes (21). We prepared complexes from purified rabbit antibodies radiolabeled with ^{125}I and either OR or ASOR (each either unlabeled or ^{131}I-labeled) and studied the behavior of light (3–4 Ab:1–2 Ag), medium (4–6 Ab:2–3 Ag), and heavy (6–7 Ab:3–4 Ag) complexes. The clearance curves and hepatic uptake data are presented using ^{125}I radioactivity. If the antigen label (^{131}I) was followed instead of antibody (^{125}I), the results were similar.

Whereas light OR complexes were cleared poorly from the circulation, resembling the poor clearance described for other small immune complexes in mice, light ASOR complexes were removed rapidly (Fig. 5). Human HAG, an Fc receptor blocking agent, had no effect on the clearance of the light ASOR complex, whereas a large dose of ASOR markedly inhibited its clearance (Fig. 5). The hepatic uptake of the ASOR complex was 80% suppressed by the blocking dose of ASOR, whereas aggregated gamma globulin had only a minimal effect (Table IIA).

Medium OR complexes were cleared faster than light OR complexes and more were found within the liver at 1 h after injection (Table IIB). Furthermore, the clearance and hepatic uptake of the OR complexes could be markedly suppressed by a prior injection of HAG, which indicates that this clearance was probably Fc receptor mediated (Table IIB). With medium ASOR complexes uptake was similarly accelerated, but there was evidence that both galactose and Fc receptors were involved. Aggregated gamma globulin caused a 35% reduction in hepatic localization of the ASOR complex, and ASOR caused a 62% reduction, which suggests that about two-thirds of the complexes were cleared via the galactose receptor and one-third via an Fc receptor. Galactose-substituted Ficoll also inhibited the hepatic uptake of the complex.

As the size of the complexes increased, the Fc portion of the antibody had a greater influence on the behavior of the complex. About 55% of heavy rabbit OR complexes remained in the circulation at 1 h. As expected, aggregated gamma globulin markedly inhibited this clearance (Fig. 6 and Table IIC). A prior injection of ASOR also diminished the amount of complex within the liver, but this was probably due to a reduction in complex size by antigen excess, because asialofetuin and asialo-ceruloplasmin, both blockers of galactose-mediated uptake, had very little effect on the clearance and hepatic uptake of these OR complexes.

The heavy ASOR complexes demonstrated rapid in vivo clearance, with only 30% of the complexes remaining in the blood at 30 min. As with the medium weight ASOR complexes, the fate of the heavy ASOR complexes was influenced to a large extent by a prior injection of aggregated gamma globulin. This led to a flattening of the clearance curve and to a 39% decrease in the uptake of these complexes by the liver. Moreover, ASOR markedly inhibited the clearance and uptake of the ASOR complexes, probably by blocking galactose receptors on hepatocytes as well as by establishing a state of antigen excess that would result in smaller complexes. Even if a blocking dose of ASOR decreased the size of the complexes from heavy

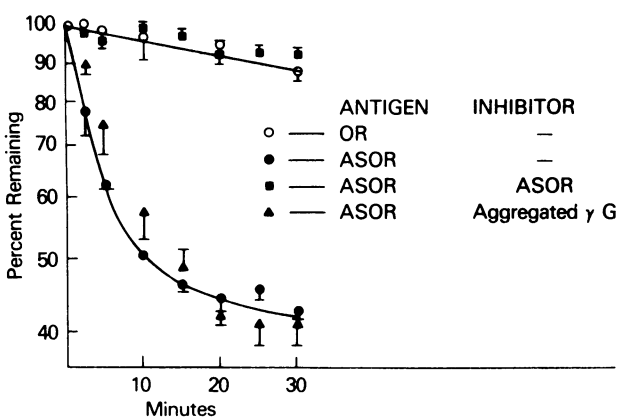

FIGURE 5 The effect of antigen and a prior injection of specific inhibitors on the blood clearance of light rabbit soluble complexes. For specific antigen and inhibitor see table within figure. ○, n = 2; ●, n = 4; ■, n = 3; ▲, n = 3. Cross bars represent mean±SEM.

TABLE II

Effect of Antigen on the Hepatic Uptake of Similarly Sized Soluble Immune Complexes with Rabbit Anti-OR Antibody

			Antigen				
			ASOR			OR	
	Inhibitor*	n	Liver uptake‡	Suppression§	n	Liver uptake‡	Suppression§
			%			%	
A Light complexes	—	4	62±1.7	0	2	18±5	0
	HAG	3	52±3.0	16		ND	
	ASOR	3	13±1	80		ND	
B Medium complexes	—	5	69±2.1	0	3	42±2.5	0
	HAG	3	45±1.3	35	3	5.8±1.6	86
	ASOR	3	26±0.9	62	3	ND	
	Galactose-Ficoll	2	45±7	35		ND	
C Heavy complexes	—	4	75±2.6	0	3	45±0.7	0
	HAG	3	46±6	39	5	5.9±1.3	87
	ASOR	4	38±3.1	49	4	17±1.8	62
	Asialofetuin	3	46±1.8	39	2	40±3	11
	Asialo-ceruloplasmin	2	54±3	28	2	56±4.5	0
	OR	2	39±3	48		ND	

* The specific inhibitor in a dose of 2–3 mg was injected 15 min before injection of the ^{125}I-immune complex.

‡ Liver uptake represents a percentage of injected ^{125}I-immune complex at 30 min for ASOR complexes and 60 min for OR complexes.

§ Suppression represents: (expected − observed)/expected × 100.

to light, however, the hepatic uptake of the resulting complexes was still less than the uptake of light weight ASOR complexes (38 vs. 62%, Table II). This decrease in uptake is most consistent only with the blocking of galactose receptors by ASOR, which would thus prevent uptake. The unrelated antigens asialofetuin and asialo-ceruloplasmin, which would have no effect on the size of the complexes, had intermediate inhibitory effects on the in vivo fate of the ASOR complexes (Fig. 6). Thus it appears that although half of the heavy complexes were removed from the serum by binding to Fc receptors, the other half were removed by binding to galactose receptors.

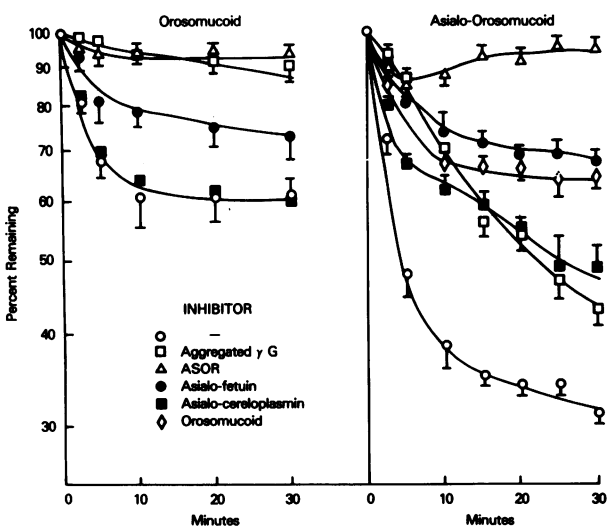

FIGURE 6 The effect of antigen and a prior injection of specific inhibitors on the blood clearance of heavy rabbit soluble complexes. Panel A, OR complexes; panel B, ASOR complexes. For specific inhibitors, see table within figure. ○, n = 3–4; □, n = 3–5; △, n = 4; ●, n = 2–3; ■, n = 2; ◇, n = 2. Cross bars represent mean±SEM.

Immune clearance and hepatic uptake of ceruloplasmin complexes. Soluble complexes made with rabbit antiasialo-ceruloplasmin and either intact ceruloplasmin or asialo-ceruloplasmin behaved similarly to the rabbit OR and ASOR complexes. All three sizes of the asialo-ceruloplasmin complexes were cleared more rapidly than the complexes of the same size containing intact ceruloplasmin. This difference was most clearly apparent with the light complexes. At 20 min after injection, 79% of the ceruloplasmin complexes remained in the circulation, compared with only 44% of the asialo-ceruloplasmin complexes. Correspondingly, 30% of the injected ceruloplasmin complexes were found within the liver at 30 min, whereas with the asialo-ceruloplasmin complexes this value was 64%. A prior injection of ASOR (which had no effect on complex size) in a dose sufficient to block most of the galactose receptors reduced the hepatic uptake of the light asialo-ceruloplasmin complexes from 64 to 30%; it had a negligible effect on the disposition of heavy ceruloplasmin complexes.

Autoradiography. After injection of radioactive rabbit OR complexes, the localization of the complexes within the liver is primarily within the sinusoidal cells (Fig. 7A, arrows), which implies that these complexes are removed from the circulation preferentially by Fc receptors. In contrast, rabbit ASOR complexes show both sinusoidal and hepatocyte localization (Fig. 7B), which confirms the inhibition studies that suggest that these complexes are taken up both by galactose receptors on hepatocytes and Fc receptors on Kupffer cells.

Cell separation studies. To document better the fate of the complexes within the liver, we separated liver cells into parenchymal (hepatocyte) and nonparenchymal (Kupffer and endothelial cell) fractions after injecting a dose of the radiolabeled complexes. Using this technique of cell separation, ASOR was taken up primarily by hepatocytes (Table III), whereas mouse antidinitrophenyl model immune complexes were primarily found within Kupffer cells. Goat ASOR complexes were, as expected, primarily taken up by hepatocytes, (nonparenchymal/hepatocyte cell ratio: 0.51) (Table III). Because goat OR complexes were poorly removed from the circulation, they were not studied. Because rabbit complexes have Fc pieces recognized by the Fc receptor of mouse, the rabbit complexes could localize to either the parenchymal (galactose-mediated) or nonparenchymal (Fc-mediated) fractions. Rabbit OR complexes demonstrated marked nonparenchymal cell uptake (nonparenchymal/hepatocyte ratio: 32) in contrast to rabbit ASOR complexes of similar size (ratio: 2.26, 2.86). As the ASOR complex size decreased there was an increased tendency for the complexes to be taken up by hepatocytes (ratio 1.66, 0.61 for light complexes).

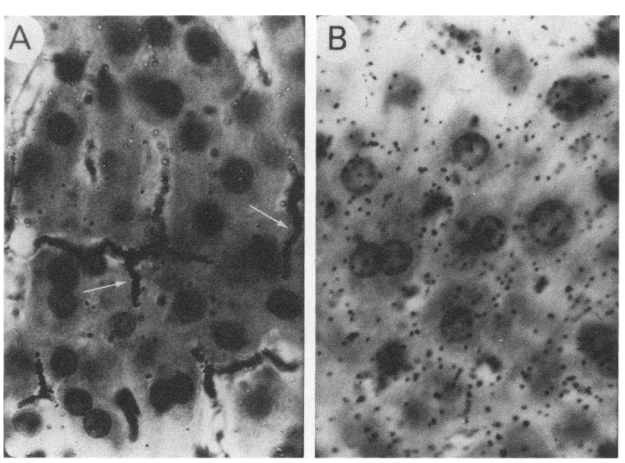

FIGURE 7 (A) Autoradiograph of the liver 60 min after injection of rabbit OR complexes. ×400. (B) Autoradiograph of the liver 20 min after injection of rabbit ASOR complexes. ×400. (See text for details.)

TABLE III

Distribution of Radiolabeled-Immune Complexes between Hepatocytes and Nonparenchymal Cells after Liver Cell Separation

Immune complex			Inhibitor	Ratio of radioactivity*
Antibody	Antigen	Size		
—	^{131}I-ASOR	—	—	0.44
^{125}I-rabbit	OR	Heavy	—	32, 38, 29
^{125}I-goat	ASOR	—	—	0.51
^{125}I-rabbit	ASOR	Light	—	1.66, 0.61
^{125}I-rabbit	ASOR	Heavy	—	2.26, 2.86
^{125}I-rabbit	ASOR	Heavy	HAG	0.48
^{125}I-rabbit	ASOR	Heavy	ASOR	5.6

* Radioactivity per nonparenchymal cell × 27/radioactivity per hepatocyte (see text for details).

Because the heavy rabbit ASOR complexes were recognized by both cell types, inhibition studies were done to see if a change in the ratio would result. After blockade of Fc receptors with aggregated gamma globulin, the nonparenchymal cell/hepatocyte ratio of heavy rabbit ASOR complexes fell to 0.48, which indicates that a greater percentage of these complexes was now being taken up by hepatocytes. Conversely, with ASOR blockade, more complexes could be found within Kupffer cells (Table III). These data substantiate the in vivo competition experiments by demonstrating the different fate of complexes varying only in the antigen.

DISCUSSION

Immune complexes are important mediators of inflammation (28). Their effector functions appear to reside within the Fc pieces of their antibodies, and the inflammation is thought to be secondary to complement activation and macrophage stimulation. It has been shown repeatedly that the reticuloendothelial (mononuclear phagocyte) system is capable of modulating circulating immune complexes by mediating their removal from the circulation (6, 29). What remains unclear, however, is why inflammation due to immune complexes localizes in particular tissues in different illnesses. If a complex escapes detection by the reticuloendothelial system, the factors influencing its inflammatory potential at certain sites within the body are largely unknown.

We have shown in these experiments that antigen can have a predominant role in determining the fate of an immune complex in vivo. These experiments took advantage of the finding that several cell types, including fibroblasts, hepatocytes, and mononuclear phagocytes, have receptors specific for terminal sugars of the carbohydrate side chains on glycoproteins (14).

It seems probable that antigens other than those we tested can bind to cells via terminal sugars or indeed other structural features and thereby lead to the binding of complexed immunoglobulins to receptor-bearing tissues. Since it appears that there is a definite period of time after an immune complex has bound onto a cell surface and before it has been internalized (30), these complexes might be able to fix complement and thus initiate a series of events that might result in localized inflammation. This process would appear to be capable of subverting a major goal of the immune system, the orderly disposal of foreign substances.

There are several ways an immune complex might deposit in the tissue independent of Fc receptors. Small complexes consisting of dimers and trimers of IgG are not readily removed by the reticuloendothelial system, presumably because of inadequate aggregation of the Fc pieces. Larger complexes (4–6 IgG molecules) are rapidly removed. Since the small complexes also fix complement poorly, it seems that if tissue injury is to occur via these complexes, ways must exist for them to bind to cell surfaces, where their effector function would be more efficient (31). One way might be by passive diffusion, but this would not explain the diversity of tissues involved in complex-mediated injury. A second way is that the charge characteristics of the complex could cause it to bind preferentially in some locations. This has been demonstrated using as antigens proteins to which haptens have been conjugated (32, 33) and using cationic antigens that bind to the anionic glomerular basement membrane (34). A recently described third way postulates that the carbohydrate on the immunoglobulin itself influences where the complex will bind (35, 36). A fourth way, which seems more specific, is that the antigen might lead to complex deposition at sites with receptors specific for the antigen itself.

In only a few of the immune complex-mediated diseases is the antigen known, and in even fewer is it well defined. The diseases most amenable to study are those caused by microorganisms. It is known, for example, that certain types of malaria may be complicated by an immune complex-mediated glomerulonephritis (37), and that in chronic schistosomiasis high levels of circulating immune complexes are found (38, 39). Antigens released from these organisms are probably of cell membrane origin and therefore probably have numerous carbohydrate chains that might be recognized by certain cells. Excreted factors from *Leishmania donovani* that are known to be antigenic have a glycoprotein structure that reacts with concanavalin A, which suggests mannose moieties available for binding to receptors (40, 41). In addition, many bacterial cell walls are known to be compounds of peptidoglycan with exposed sugar side chains (42). These antigens in an immune complex might be able to direct that complex to tissues where they could bind to specific receptors. The aggregated Fc pieces of the tissue-bound complex might then initiate an inflammatory response.

In addition to being capable of directing complexed immunoglobulin to a certain tissue, sugars on an antigen may influence how it is processed after binding. Since receptors exist on mononuclear phagocytic cells for the terminal sugar on glycoproteins (mannose or *N*-acetyl-glucosamine) and for the Fc piece of IgG, IgG complexed with antigen may be handled differently by the cell depending on which receptor recognizes it. For instance, it is known that the polysaccharide of *Schistosoma mansoni* eggs localize within macrophages and persists within the cell for extended periods of time (43). It is conceivable that the fate of this antigen might be different if it were taken up into a macrophage by a sugar receptor instead of as an immune complex via an Fc receptor. One route may be degratory, another immunogenic.

Furthermore, it is known that immune complexes can modulate an antibody response to the antigen within the complex (44); that this modulation may involve T cells; that T cells can bind concanavalin A, which suggests that mannose residues are exposed on their outer surfaces (45); and that some macrophages have mannose receptors (14). It is reasonable to speculate, therefore, that if carbohydrate-containing antigens interact with macrophage receptors, they may alter macrophage-T cell interaction and hence immune responses (46).

Finally, since there are diseases in which circulating immune complexes appear to have a pathogenetic role and diseases in which they do not, it is probable that complexes can be either destructive or neutral. In this context, we propose that the antigen may in part determine the character of a complex.

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