Complement Depletion Accelerates the Clearance of Immune Complexes from the Circulation of Primates

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Abstract. Binding of immune complexes (IC) to erythrocytes in vitro is the result of interaction between C3b sites on the IC, and complement receptors type I (CR_1) expressed on primate erythrocytes. Recent evidence indicates that primate erythrocytes can also rapidly bind large, preformed IC in vivo. This study was undertaken to determine if the binding of IC to baboon erythrocytes in vivo is complement dependent and to examine the effect of complement depletion on IC clearance from the circulation. The results indicate that complement depletion in vivo reduced the binding of IC to erythrocytes. There was relatively little binding of IC to leukocytes in both the complement-depleted and complement-repleted condition. Thus, the majority of IC not bound to erythrocytes remained free in the plasma and, consequently, IC infusion during the complement-depleted state resulted in increased plasma IC concentrations. This was associated with a rapid disappearance of IC from the circulation. By contrast, in the normal or complement-repleted state, a large fraction of the IC became bound to erythrocytes during IC infusion, which resulted in lower plasma IC concentrations. Under these conditions, a more gradual rate of disappearance of IC from the circulation was observed. The relatively abrupt clearance of IC from the circulation in the complement-depleted state could not be accounted for by increased hepatic or splenic uptake. These data indicate that, in contrast to previous studies in nonprimates, complement depletion in primates results in accelerated removal of IC from the circulation. This suggests that factors such as hypocomplementemia and deficient expression of erythrocyte CR_1, which are known to occur in certain IC-mediated diseases, may promote IC uptake by organs vulnerable to IC-mediated injury.

Introduction

It is generally believed that complement plays little or no role in the clearance of soluble immune complexes (IC)[1] from the circulation (1). This conclusion is based mainly on complement depletion (complement-D) studies in rabbits (2) and mice (3). However, recent evidence suggests that primates possess a mechanism for the clearance of IC which differs from nonprimates (4). Primate erythrocytes, but not nonprimate erythrocytes, bear complement receptor type I (CR_1) which can bind C3b (5). Large, preformed IC rapidly become bound to primate erythrocytes in vivo (4). Once bound, most erythrocyte-borne IC are deposited in the liver. The erythrocyte then returns to the circulation apparently able, once again, to participate in this erythrocyte-IC clearing mechanism (4).

The evidence that erythrocytes participate in the clearance of IC from the circulation prompted the reexamination of the role of complement in IC clearance in primates. The study presented here was undertaken to determine if the binding of IC by baboon erythrocytes in vivo is complement-dependent, and to determine if complement-D resulted in altered IC clearance. The results indicate that IC binding by erythrocytes in vivo is indeed complement dependent. In addition, an increase in the rate of IC removal from the circulation was observed in complement-D primates.

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1. *Abbreviations used in this paper:* CVF, cobra venom factor; complement-D, complement depleted (CVF experiments) or complement defective (heparin experiments); complement-N, normocomplementemic (pre-heparin infusion) or complement repleted (plasma reinfusion); CR_1, complement receptor type I; IC, immune complex; PEG, polyethylene glycol.

Methods

Preparation of IC. The IC used in these experiments were prepared as described previously (4). Briefly, ^{125}I-bovine serum albumin (BSA) was added at equivalence to heat-inactivated rabbit anti-BSA serum. After incubation for 30 min at 37°C, the mixture was held at 4°C for 48–72 h. The resulting precipitate was then solubilized in fivefold excess of unlabeled BSA for 4 h at 37°C. The solution was centrifuged at 100 *g* for 20 min. The supernatant was then collected and utilized as the source of IC.

Complement depletion. Complement inhibition was achieved by infusing heparin (from porcine intestines, Elkins-Sinn, Inc, Cherry Hill, NJ); complement depletion was achieved by infusing *Naja haje* CVF (Cordis Laboratories, Miami, FL), as described below. These states are collectively referred to as complement-D. In order to monitor functional complement achieved by cobra venom factor (CVF), plasma samples taken during the experiment were used as the complement source in an in vitro IC to erythrocyte binding assay, as described elsewhere (4).

Experimental protocol. Seven primates were studied. Each animal was studied in the complement repleted (complement-N) and in the complement-D state. Two baboons and one Rhesus monkey received heparin (6,300 U/kg). One baboon received heparin at a lower dose (2,100 U/kg). Three baboons received CVF (225 U/kg). The surgical preparation and angiographic procedures used for catheter placement have been described elsewhere (4). In brief, for the infusion of IC, a catheter was placed via the common femoral artery into the ascending aorta just above the aortic valve. To measure clearance of IC from arterial blood and across liver and kidney, catheters were inserted in the abdominal aorta at a point midway between the hepatic and renal arteries (to monitor arterial blood IC concentration), the renal vein (to monitor IC uptake by kidney), and the hepatic vein (to monitor IC uptake by liver). In two CVF experiments, a catheter was also introduced directly into the portal vein (to monitor IC uptake by spleen plus gut and to monitor delivery of IC to liver. In one CVF experiment, catheters were placed only in the ascending aorta and the abdominal aorta. After catheter placement, autologous ^{51}Cr-labeled erythrocytes in all experiments were infused and allowed to circulate for a minimum of 20 min. Before IC infusion, blood samples (1 ml) were drawn into heparin-coated syringes and placed in tubes on ice as a zero time sample.

Basic protocol for studying IC clearance. IC (0.22–0.86 mg protein in 6–7 ml) were infused at a constant rate over 2 min into the aortic arch catheter, and blood specimens (usually 1 ml) were obtained from the arterial and the hepatic, renal and portal vein catheters at frequent intervals (each 20–30 s for 2 min, then at 3, 4, 5, 10, 15, and 30 min). This protocol was then repeated after the animal was rendered complement defective by heparin infusion ("heparin experiments") or complement repleted by infusion of autologous plasma ("CVF experiments"). The details of the protocols of the heparin experiments and the CVF experiments are given below.

Heparin experiments. IC clearance in each animal was examined first under normal (complement-N) conditions. Heparin was then infused through the aortic arch catheter over 10 min and allowed to circulate a minimum of 10 min. At this point, the IC clearance protocol was repeated using the same dose of IC.

There is evidence that another sulfated compound, trypan blue, binds to primate erythrocyte CR_1 (5). In order to determine if heparin also could act to interfere with IC binding by blocking CR_1, the following experiment was performed. Initially, IC together with baboon serum were incubated in the presence or absence of heparin in order to generate IC-C3b. Subsequently, baboon erythrocytes with or without heparin were added and the reaction mixtures were incubated to permit binding of IC-C3b to CR_1. The result indicated that, under these experimental conditions, heparin acts by preventing the generation of C3b on IC rather than by blocking the binding of IC-C3b to erythrocyte CR_1 (Table I).

CVF experiments. Each baboon underwent plasmapheresis equal to ~40% of plasma volume 1 to 2 wk before the experiment. The plasma removed was replaced with ~1.5 times the volume of saline. The plasma was frozen at −20°C until 1 h before the experiment when it was thawed. 24 h before the experiment, the CVF was infused intravenously over a 10-min period into an animal lightly anesthetized with ketamine. After recovering from the anesthesia, the animals appeared entirely well. On the day of the experiment, the basic IC clearance protocol was performed, first in the complement-D state. Autologous plasma was then infused over 20 min to partially replete serum complement levels. The basic IC clearance protocol was then repeated, using the same dose of IC.

Processing of blood samples. Each blood sample was drawn into a syringe and immediately transferred to a tube on ice. The procedure for assessing IC binding to erythrocytes was described previously (4). Briefly, 0.5 ml of whole blood was placed on 2 ml of cold 65% percoll (density equals 1.10 g/ml, Sigma Chemical Co, St Louis, MO) and centrifuged at 350 *g* for 15 min. Under these conditions, an "erythrocyte fraction" (erythrocytes and IC bound to erythrocytes) migrated to the bottom of the tube, while the supernatant fluid contained percoll, leukocytes, and unbound IC. The ^{125}I cpm of the erythrocyte fraction and of the supernatant fluid were then determined in a gamma

*Table I. Heparin Blocks the Generation of C3b on IC Rather than the Binding of IC-C3b to Erythrocyte CR_1**

Group	Heparin added at: Step 1	Heparin added at: Step 2	^{125}I-cpm±1 SD: Erythrocyte-bound	^{125}I-cpm±1 SD: Unbound	Percent binding
A	—	—	1,320±125	2,144±233	38.2
B‡	—	—	158±4	3,099±156	4.8
C	+	—	285±27	3,042±155	8.6
D	—	+	1,252±5	1,924±25	39.2

* The experiment was carried out in duplicate tubes, using heparin at the dose employed in the in vivo studies (90 U/ml) as follows:

Step 1. A reaction mixture consisting of 10 μl of fresh baboon serum, 50 μl of IC, and 40 μl of PBS (±heparin) was incubated for 10 min at 37°C.

Step 2. 50 μl of packed baboon erythrocytes and 13.5 μl of either PBS (groups A to C) or heparin (group D) was added and the reaction mixture was incubated for an additional 10 min at 37°C. After this incubation period, the reaction mixtures were centrifuged over 65% percoll gradients, and the ^{125}I-cpm bound to erythrocytes (pellet) or unbound (supernatant fluid) was determined.

‡ As a negative control, heat-inactivated serum (56°C for 30 min) was substituted for fresh serum in group B.

Data are representative of two experiments.

scintillation counter, and the percentage of IC bound to erythrocytes was calculated as described previously (4).

To determine IC binding to the leukocytes, whole blood was mixed with an equal volume of cold phosphate-buffered saline (PBS), placed on top of iced 30% percoll, and centrifuged at 350 *g* for 15 min. Under these conditions, both leukocytes and erythrocytes penetrate the percoll and form a pellet. The percentage of IC bound to erythrocytes plus leukocytes was then calculated. The percentage of IC bound to erythrocytes alone (65% percoll) was subtracted from the percentage of IC bound to erythrocytes plus leukocytes (30% percoll) in order to determine the percentage of IC bound to leukocytes.

Analysis of the clearance of IC from arterial blood. The period commencing immediately after the cessation of the IC infusion was chosen for analysis because after that time point, the blood IC level is affected only by the rate of IC clearance from the blood. Before that time, the blood IC level is affected by both the rate of IC infusion and the rate of IC clearance from the blood. Blood IC levels were taken as proportional to ^{125}I levels. This is a valid assumption, since ultracentrifugation studies show that virtually all of the ^{125}I label in the IC preparation is contained within IC (4). In addition, relatively little metabolism of IC occurs in vivo during the course of the experiment, since polyethylene glycol (PEG) precipitation of plasma specimens taken after IC infusion show that the majority of the ^{125}I is associated with IC (see Results). Relatively little release of free ^{125}I occurs during the course of the experiment since less than 1% of the ^{125}I dose is excreted in urine as measured in samples that are obtained at the conclusion of the experiment.

To compare the blood IC levels after the first dose of IC to the blood IC levels after the second dose of IC, it was necessary to correct for the residual level of radioactivity in blood after the first IC infusion. This was done by subtracting the whole blood ^{125}I cpm/ml obtained just before the second IC infusion from each of the measurements of ^{125}I cpm/ml made on subsequent blood samples. This is a valid correction, since 30 min after the infusion of the first IC dose the blood levels of radioactivity are low and stable (see Figs. 4 and 5). In addition, the ^{125}I cpm/ml of whole blood were expressed as the percentage of the peak level of ^{125}I cpm that was achieved during the given IC infusion. This was done to correct for minor variations in the rate of IC infusion or changes in blood volume that might occur between the first and the second IC dose. This normalization of the data also permitted the comparison of IC clearance rates between experiments. As noted above, the dose of IC varied from experiment to experiment because there are variations in the amount of IC solubilization during IC preparation.

Calculation of hepatic vs. nonhepatic IC uptake. Hepatic uptake of circulating IC was estimated as follows:

Absolute hepatic IC uptake over the period t_1 to t_2:

$$= \mathrm{HF}[\mathrm{IC(A)} - \mathrm{IC(HV)}], \quad (1)$$

where IC(A) equals mean arterial blood IC concentration over the period t_1 to t_2, and equals mean ^{125}I cpm/ml of arterial blood over the period t_1 to t_2 determined from the area under the curve of a plot of ^{125}I cpm/ml values vs. time; where IC(HV) equals mean hepatic vein blood IC concentration over the period t_1 + 20 s to t_2 + 20 s. The reason for taking the later time period for the hepatic vein samples is that the vascular transit time for liver is ~20 s, under the present experimental circumstances (6). Thus, the hepatic vein blood sample that corresponds to the arterial blood sample is obtained ~20 s after the arterial sample. This adjustment is important under conditions in which the arterial blood concentration is changing rapidly; and equals mean ^{125}I cpm/ml of hepatic vein blood over the period t_1 + 20 s to t_2 + 20 s determined from the area under the curve of a plot of ^{125}I cpm/ml values vs. time; where HF equals total hepatic blood flow over the period t_1 to t_2, and equals 0.22 times cardiac output over the period t_1 to t_2. The coefficient 0.22 is an average value taken from microsphere distribution data in the baboon and the dog (4). Cardiac output per minute was arbitrarily taken as equal to blood volume (7% of body weight plus plasma infused [CVF experiments, only]).

Fractional hepatic IC uptake over the period t_1 to t_2:

$$= [\mathrm{IC(A)} - \mathrm{IC(HV)}]/\mathrm{IC(A)}. \quad (2)$$

Portal vein blood samples were collected in two of the CVF experiments but could not be collected in the heparin experiments because of the risk of hemorrhage that was associated with the placement of the portal vein catheter. Thus, hepatic IC uptake was not corrected for portal vein flow, as was done in our previous work (4). Thus, the designation "hepatic uptake" is actually uptake by liver plus spleen plus gut plus pancreas. Nevertheless, >90% of splanchnic IC uptake is hepatic IC uptake (4). Thus, it is appropriate to refer to IC uptake in the splanchnic circulation as hepatic uptake.

To calculate hepatic IC uptake from the erythrocyte fraction vs. the plasma/buffy coat fraction of blood, Eqs. 1 and 2 were used, except that the ^{125}I cpm of the erythrocyte or plasma/buffy coat fraction of blood was substituted for the whole blood ^{125}I concentration.

Total IC uptake from the circulation over the period t_1 to t_2:

$$= \mathrm{BV}[\mathrm{IC(A)}_{t_1} - \mathrm{IC(A)}_{t_2}], \quad (3)$$

where $\mathrm{IC(A)}_{t_1}$ and $\mathrm{IC(A)}_{t_2}$ equal IC concentration in arterial blood at time t_1 and t_2, respectively, and equal ^{125}I cpm/ml of whole blood at time t_1 and t_2, respectively. BV equals blood volume, taken as 7% of body weight plus plasma infused (CVF experiments only).

Thus absolute nonhepatic IC uptake over the period t_1 to t_2:

$$= \text{Eq. 3} - \text{Eq. 1}. \quad (4)$$

Calculation of renal IC uptake. The renal vascular transit time is ~3 s (6). Thus, to assess fractional IC uptake by kidney, it is valid to compare arterial samples with renal vein samples that were obtained with about a 3-s delay. The delay in obtaining renal vein samples compared with arterial samples was ~3 s. Accordingly, to assess renal IC uptake, the following calculation was applied:

Fractional renal IC uptake over the period t_1 to t_2:

$$= [\mathrm{IC(A)} - \mathrm{IC(RV)}]/\mathrm{IC(A)}, \quad (5)$$

where IC(RV) equals renal vein IC concentration over the period t_1 to t_2, and equals mean ^{125}I cpm/ml of renal vein blood over the period t_1 to t_2, determined from the area under the curve of a plot of ^{125}I cpm/ml of blood vs. time.

Analysis of IC in plasma. To determine if the ^{125}I cpm not bound to cells represented free ^{125}I or ^{125}I-BSA, rather than IC, the supernatant fluids from 30% percoll gradients were precipitated with 20% PEG as previously described (4). To assess the size of IC in plasma, in selected experiments plasma samples were subjected to centrifugation on isokinetic sucrose gradients, as previously described (7).

Statistics. All mean values are shown ±1 SE of the mean. Comparisons were analyzed by paired *t* test.

Results

Effect of complement-D on IC binding by erythrocytes. CVF treatment reduced or abolished the capacity of plasma from the treated primates to support IC binding by erythrocytes in vitro (Table II). CVF-treated baboons also exhibited a marked impairment in the binding of IC by erythrocytes in vivo as shown by the representative study in Fig. 1 and the data summary presented in Table III. Reinfusion of autologous plasma into CVF-treated baboons partially restored the capacity of erythrocytes to bind IC in vivo (Fig. 1). Treatment with high doses of heparin also markedly impaired binding of IC by primate erythrocytes in vivo, as shown by the representative study in Fig. 2. This inhibition of erythrocyte IC binding occurred at heparin concentrations that have been shown to inhibit complement activation (8, 9) but did not occur at a lower dose of heparin (Fig. 3). Both the low and high doses of heparin were far greater than that needed for anticoagulation. These data strongly suggest that the binding of large IC by primate erythrocytes in vivo is complement dependent since two pharmacologically distinct agents, but having in common the capacity to deplete or inhibit complement activity, both markedly suppressed IC binding to erythrocytes.

Hemolytic complement assays were also performed in some experiments (Table II). Hemolytic complement assays were found to be a less reliable index of complement activation in the baboon because CH_{50} levels < 55 U were commonly observed, even in normal baboons. At CH_{50} levels < 55 U, the assay becomes less accurate. We suggest that this is the reason for the inconsistent relationship between IC-to-erythrocyte binding and CH_{50} levels, from experiment to experiment.

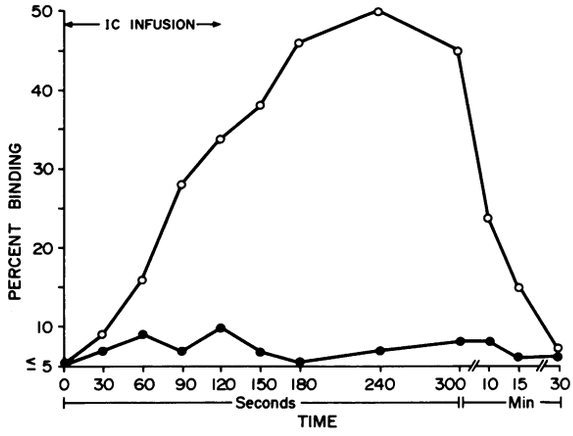

Figure 1. Effect of complement depletion by CVF on IC binding to erythrocytes in vivo (exp. CVF #2). IC were infused into a baboon over a 2-min period, and periodic arterial blood samples were obtained. The blood samples were centrifuged on percoll gradients to separate erythrocyte-bound IC from unbound IC. At 30 min post IC infusion, autologous plasma was infused into the circulation. The IC clearance experiment was then repeated. The data are expressed as the percentage of IC bound to erythrocytes before (●), or subsequent to (○) plasma infusion.

Table II. Effect of Complement-D on Serum Hemolytic Complement Levels and on the Capacity of Plasma to Support the Binding of IC to Erythrocytes In Vitro

Experiment number	Source of plasma*	Hemolytic complement assay‡	IC to erythrocyte binding§
		CH_{50} U	%
CVF-1	Before CVF	127	82
	Post CVF	60	4
	Post plasma reinfusion	102	
CVF-2	Before CVF	116	75
	Post CVF	0	6
	Post plasma reinfusion	12	
CVF-3	Before CVF	60	83
	Post CVF	0	4
	Post plasma reinfusion	1	
Heparin	Before heparin	44	‖
	Post heparin	34	

* Plasma was obtained immediately before the infusion of CVF or heparin (before CVF or heparin), immediately before IC infusion (post CVF or post heparin), and 20–30 min after reinfusion of plasma (post plasma reinfusion).

‡ Under the experimental conditions employed, hemolytic complement levels (CH_{50}) < 55 U are considered to be subject to a high degree of experimental error.

§ The assay employed to assess the capacity of plasma to support the binding of IC to erythrocytes in vitro is described in detail elsewhere (4).

‖ The results of IC-to-erythrocyte binding in vivo are shown in Table III.

Table III. IC Binding to Erythrocytes In Vivo at Time of Peak IC Concentration in Blood

Experiment number	% IC bound to erythrocytes	
	Complement-D	Complement-N
CVF #1	10.9	34.5
CVF #2	10.7	38.3
CVF #3	(See fig. 6)	(See fig. 6)
Hep #4	12.0	65.7
Hep #5	38.2	93.6
Hep #6	4.4	44.2
Mean	15.2±5.9*	55.3±11

* $P < 0.01$ compared with IC bound to erythrocytes during complement-N.

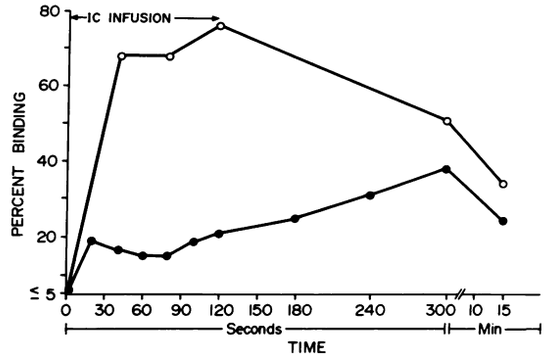

Figure 2. Effect of complement inhibition by heparin on IC binding to erythrocytes in vivo (exp. heparin #4). IC were infused into a normal baboon over a 2-min period, and periodic arterial blood samples were obtained. The blood samples were centrifuged on percoll gradients to separate erythrocyte-bound IC from unbound IC. At 30 min post IC infusion, heparin (6,300 U/kg) was infused into the circulation. The IC clearance experiment was then repeated. The data are expressed as the percentage of IC bound to erythrocytes before (○), or subsequent to (●) heparin infusion.

Despite these limitations, the CH_{50} data are consistent with the interpretation that complement inhibition was induced in the complement-D portions of the experiments.

State of IC in blood which are not bound to erythrocytes. Data presented in Table IV indicate that most of the ^{125}I cpm not bound by erythrocytes were actually free in the plasma rather than bound to leukocytes. However, a somewhat greater proportion of IC was bound to leukocytes in the complement-N state compared with the complement-D state. To assess the extent to which unbound ^{125}I cpm in plasma represented IC,

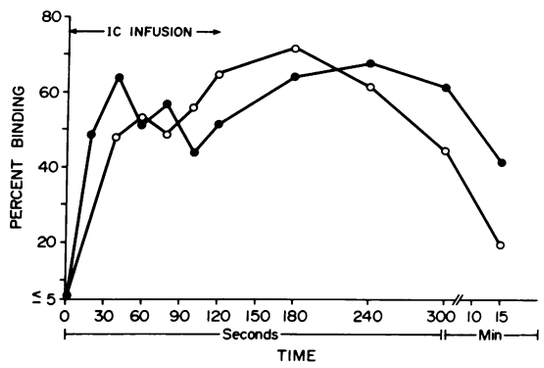

Figure 3. Effect of low dose heparin infusion on IC binding to erythrocytes in vivo. The experimental protocol is identical to that described in the legend for Fig. 2, with the exception that the dose of heparin was 2,100 U/kg. The data are expressed as the percentage of IC bound to erythrocytes before (○), or subsequent to (●) heparin infusion. Under these conditions, heparin infusion had little effect on IC binding to erythrocytes and on IC clearance from the blood.

the plasma was treated with PEG. The results showed that the majority of plasma ^{125}I cpm were precipitated by PEG, which indicated the presence of IC rather than free antigen or ^{125}I in the plasma. This was the case in the complement-N as well as in the complement-D state.

Effect of complement-D on the clearance of IC from the circulation. The clearance of IC from the arterial blood of a baboon in a representative CVF experiment is shown in Fig. 4. The concentration of IC in CVF-treated baboons reached a peak at 120 s, the time at which the infusion of IC was completed. During the 60-s interval immediately following the cessation of IC infusion, the concentration of arterial IC declined precipitously. This reduction in blood IC concentration was due to uptake of IC out of the circulation and not to hemodilution, since blood ^{51}Cr cpm/ml did not change during this period (data not shown). The rapid decline in blood IC levels during complement-D was in contrast to the handling of IC in the same baboon after partial repletion of complement activity by infusion of autologous plasma (Fig. 4). Under conditions of complement-N, arterial blood IC levels fell gradually after cessation of the IC infusion.

A representative heparin experiment is shown in Fig. 5. This experiment also shows the greater fall in arterial blood IC clearance rate in the complement-D compared with the complement-N state, in the period immediately after cessation of the IC infusions.

Table V shows the data for IC clearance from arterial blood during the period of rapid clearance in all experiments. As can be seen, the complement-D states show greater rates of IC removal from the blood. The single exception is heparin exp. 6. However, in this experiment an increased rate of IC clearance was taking place even during the period of IC infusion (IC blood levels averaged 44% lower during the period of IC infusion in the complement-D compared with the complement-N state). Thus, perhaps the reason for failing to observe an increased rate of IC clearance in the complement-D state during the period of rapid clearance in this one experiment is that most of the IC susceptible to rapid clearance may have already been removed from the circulation.

Fig. 6 shows the results of an experiment in which IC were infused as a 5-s bolus. As can be seen, the rate of disappearance of IC from the arterial blood was greater in the complement-D vs. the complement-N state but not as great as in the experiments in which the IC were infused over 120 s. In designing this experiment it was anticipated that the relatively high blood IC concentration that was achieved by bolus infusion would result in a marked difference in the IC clearance rate from blood in the complement-D vs. the complement-N state. This was not observed, perhaps because after the bolus IC infusion the initial percentage of IC bound to erythrocytes was equally low in the complement-D and in the complement-N state. Thus, initially in both states there was a large fraction of IC free in the plasma, and in both states the rates of IC clearance from the blood were comparable. Nevertheless, after

Table IV. IC Bound to Erythrocytes Vs. Erythrocytes Plus Leukocytes under Conditions of Complement-D Vs. Complement-N

Experiment number	Source of sample	% of ^{125}I-cpm bound to erythrocytes*	% of ^{125}I-cpm bound to erythrocytes plus leukocytes‡	% of ^{125}I-cpm bound to leukocytes§	% of PEG precipitable ^{125}I-cpm free in plasma‖
CVF #2					
CVF treated	90 s post IC	7	3	0	77
	120 s post IC	10	5	0	70
	150 s post IC	7	6	0	76
Post plasma reinfusion	90 s post IC	28	37	9	67
	120 s post IC	34	44	10	59
	150 s post IC	38	44	6	66
CVF #3					
CVF treated	60 s post IC	5	12	7	69
	30 min post IC	—	—	—	52
Post plasma reinfusion	60 s post IC	51	70	19	53
	30 min post IC	—	—	—	53
Heparin #6					
Before heparin	100 s post IC	44	64	20	62
	120 s post IC	52	74	22	50
	180 s post IC	58	55	0	—
Post heparin infusion	100 s post IC	4	9	5	61
	120 s post IC	7	6	0	62
	180 s post IC	12	18	6	—

* Erythrocyte-bound IC determined by centrifugation on 65% percoll gradients. ‡ Erythrocyte-bound IC and leukocyte-bound IC determined by centrifugation on 30% percoll gradients. § Leukocyte-bound IC calculated by subtracting erythrocyte-induced IC value from erythrocyte-bound and leukocyte-bound value. ‖ PEG precipitation performed on the supernatant fluid from the 30% percoll gradient. In four separate experiments, the percentage of uncomplexed ^{125}I-BSA precipitated by 20% PEG averaged 18%. The percentage of ^{125}I which was PEG precipitable in the IC preparations before infusion into the animal was 88.2±2.3% (n = 4).

30–40 s, increasing levels of IC binding to erythrocytes were now detectable under conditions of complement-N and from that point forward there was a gradual trend towards a slower IC removal rate in complement-N compared with complement-D. Thus, in this bolus infusion experiment it was possible to show a relationship between the onset of binding of IC to erythrocytes and the slowing of IC clearance from the circulation. This lends further support to the hypothesis that the effect of complement-D to accelerate the clearance of IC from the circulation is related to decreased IC-to-erythrocyte binding caused by complement-D.

Effect of repeated IC infusions in a normal baboon. Both experimental protocols for achieving complement-D involve the administration of two consecutive IC infusions in the same baboon. In order to determine if one IC infusion in a normal baboon had any effect on IC-to-erythrocyte binding or on the clearance kinetics of subsequent IC infusions, the following experiment was performed. IC, at a dose comparable with that used throughout this study, were infused over a 2-min period and the initial clearance of IC was measured over 30 min. At this point, a second identical dose of IC was administered and IC clearance was again measured over 30 min. Finally, a third IC injection was given and the IC clearance measurements were made for a third time. The results indicated that, at the dose of IC employed, there was no appreciable difference in IC to erythrocyte binding, IC clearance kinetics, or in arterial or hepatic vein ^{125}I cpm levels (either erythrocyte-bound or unbound), between the first, second, or third IC infusions (Fig. 7).

Hepatic vs. nonhepatic uptake of IC under conditions of complement-N vs. complement-D. As can be seen from Table VI, in the 60-s interval after cessation of the IC infusion (the period of rapid clearance) there is a greater amount of IC removed from the circulation in the complement-D compared with the complement-N state. Calculated hepatic IC uptake increased during complement-D. However, the calculated in-

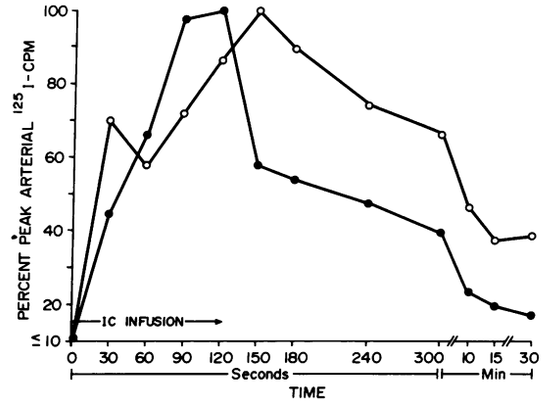

Figure 4. Effect of complement-D by CVF on the clearance of IC from the blood (CVF #2). The experimental protocol is described in the legend of Fig. 1. The data are expressed as the percentage of the peak ^{125}I cpm remaining in the circulation (including both erythrocyte-bound and unbound) either before (●), or subsequent to (○) infusion of autologous plasma.

crease in hepatic IC uptake could not account for the marked increase in the rate of IC removal from the circulation. Calculated hepatic IC uptake assumes that total hepatic blood flow is unchanged during the experiment (except for a calculated increase due to plasma infusion; see Eq. 1). However, it is clear from the increase in the absolute amount of IC removal from the circulation that no reasonable increase in hepatic blood flow (hepatic IC delivery rate) during complement-D could account for the marked increase in IC removed from the blood. Finally, there is no reason to suspect that splenic IC uptake could account for the marked increase in nonhepatic

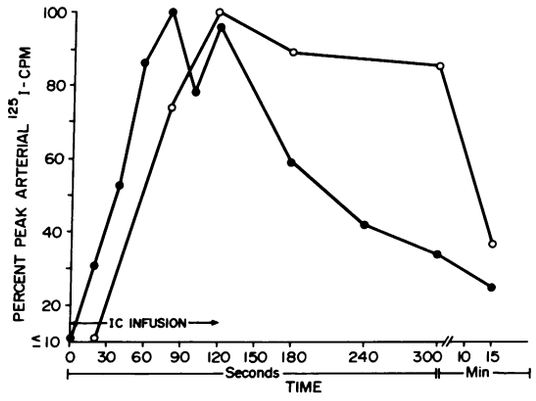

Figure 5. Effect of complement-inhibition by heparin on the clearance of IC from the circulation (heparin #4). The experimental protocol is described in the legend for Fig. 2. The data are expressed as the percentage of the peak ^{125}I cpm remaining in the circulation (including erythrocyte-bound and unbound) either before (○), or subsequent to (●) heparin infusion.

Table V. Clearance from Arterial Blood during the Period of Rapid Clearance (the 60-S Period after Cessation of IC Infusion) in Complement-N Vs. Complement-D

Experiment number	% Change in arterial blood IC concentration*	
	Complement-N	Complement-D
CVF #1	4.4	45.4
CVF #2	(−3.5)	47.2
CVF #3	12.1	28.2
Hep #4	15.7	38.5
Hep #5	42.1	60.0
Hep #6	16.4	14.6
Mean	14.5±6.3	39.0±6.5‡

* 1 − [(^{125}I-cpm at 60 s post peak)/(^{125}I-cpm at peak)] × 100.
‡ $P < 0.05$, compared with the percentage change in arterial blood IC concentration under conditions of complement repletion.

IC uptake under conditions of complement-D because, under these experimental conditions, spleen receives only ~15% of total splanchnic flow (unpublished observations based on microsphere distribution data from previously published studies in the primate). Thus, even if spleen removed all of the IC delivered to it, it could not account for the large amount of IC removed from the circulation under conditions of complement-D.

The tendency for calculated hepatic IC uptake to be increased during the period of rapid clearance in complement-D compared with complement-N was due primarily to lower IC concentration in hepatic vein blood under conditions of complement depletion compared with complement repletion (see Eq. 1). Table VII shows that the lower hepatic vein IC concentration was due to a significant increase in the fractional uptake of IC out of the plasma. The fractional rate of removal of IC from the erythrocyte fraction was not changed by complement-D. Spleen plus gut also exhibited an increase in fractional uptake of IC under conditions of complement-D. This is shown in the two CVF experiments in which portal vein blood samples were obtained. These data revealed a marked increase in fractional IC uptake, calculated from arterial/venous differences, which was the result of a marked reduction in portal vein IC concentration under conditions of complement-D. The fractional IC uptake by spleen plus gut under conditions of complement-N vs. complement-D was 5 vs. 31% (CVF #1) and 18 vs. 85% (CVF #2). Indeed, it is clear that spleen plus gut contributed, in part, to the increased calculated hepatic IC uptake noted during conditions of complement-D. However, the total contribution of spleen plus gut cannot be accurately determined without knowing the apportionment of hepatic flow between hepatic artery and portal vein, as previously described (4).

Renal uptake of IC under conditions of complement-N vs. complement-D. No consistent change was observed in renal IC

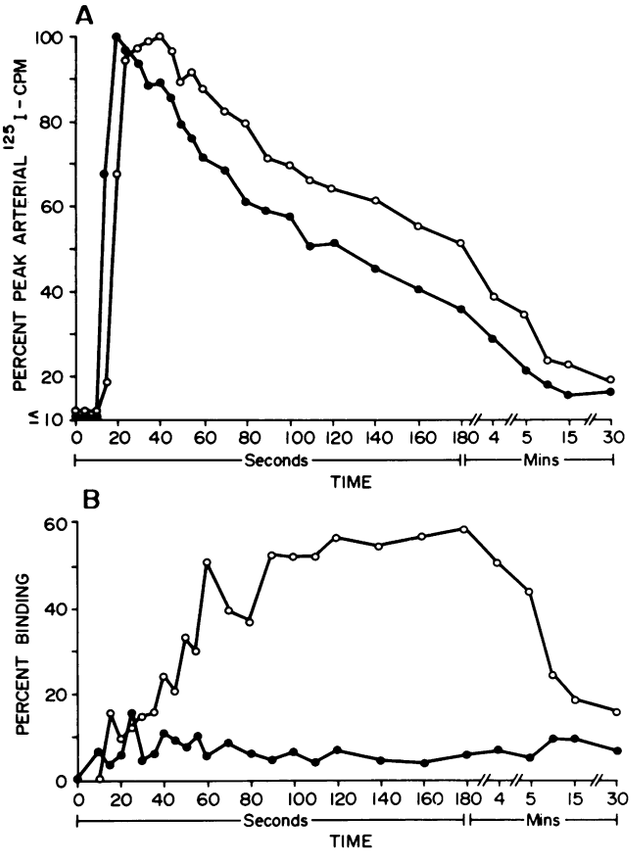

Figure 6. Clearance of IC infused as a bolus from the circulation of a CVF-treated baboon (CVF #3). The experimental protocol is as described in the legend of Fig. 1 with the following modifications: (*a*) IC were infused as a 5-s bolus, and (*b*) blood was collected at 5-s intervals during the first 60 s post IC infusion, at 10-s intervals from 60 to 120 s, and at 20-s intervals from 120 to 180 s. Additional blood samples were obtained at 4, 5, 10, 15, and 30 min post IC infusion. The data are expressed as the percent peak arterial ^{125}I cpm either before (•), or subsequent to (○) infusions of autologous plasma (*A*); and as the percent binding of IC to erythrocytes either before (•), or subsequent to (○) infusion of autologous plasma (*B*).

uptake out of the plasma/buffy coat or erythrocyte fraction of blood as a result of complement-D.

Size of IC remaining in the sera of complement-D baboons. There is evidence that primate erythrocytes bind large IC more efficiently than small IC (5). It was therefore of interest to determine if the size profile of IC remaining in the circulation after peak IC clearance was altered in complement-D baboons. Based on the data from the initial experiments, the time of 180 s after the commencement of IC infusion was chosen as the sampling time. Two baboons, one heparin treated and one CVF treated, were studied.

The size profiles of the IC used in these experiments consisted of mixed populations of very large, intermediate, and relatively small IC (Fig. 8). In both the normal and heparin-treated state, the residual plasma IC immediately after peak clearance consisted almost exclusively of relatively small IC (Fig. 8 *A*). In the CVF experiment, the percentage of small IC in the circulation at 180 s post-IC infusion was higher under conditions of complement-N vs. complement-D (Fig. 8 *B*). Complement-N may promote degradation of IC in vivo, as has been shown in vitro (10–13). The data also indicate that large IC are cleared from the circulation more rapidly under conditions of both complement-D and complement-N.

Hemodynamic monitoring and general clinical data. In the CVF experiments, under conditions of complement-D vs. complement-N, mean blood pressure was stable and ranged between 90 and 95 mmHg. In the heparin experiments, under conditions of complement-N vs. complement-D, mean blood pressure was stable and ranged between 100 and 120 mmHg. Platelet count, white blood cell count, and differential were measured before and after IC infusion in one experiment and were found to be normal and not changed by the infusion of IC. The CVF animals appeared healthy and did not appear to be affected by the CVF treatment. Similarly, the high dose heparin animals showed no ill effects during or after the heparin infusion. All animals survived in health except one CVF animal which died 24 h after the experiment because of delayed hemorrhage from the site of the portal vein catheter.

Discussion

There is evidence that, in primates, the erythrocyte plays an important role in the clearance of IC from the circulation (4). Although these studies involve a heterologous system using rabbit antibody and BSA, it should be noted that efficient IC binding to primate erythrocytes has also been demonstrated using IC probes composed of mouse antibodies (14) or human antibodies (15). IC-to-erythrocyte binding also is not specific for a given antigen, since the IC probes used in the above studies have included BSA, DNP-substituted BSA, and DNA.

Data presented here indicate that binding of IC to erythrocytes in vivo is inhibited by both heparin and CVF. Heparin in high doses inhibits the complement cascade apparently by interfering with formation or regulation of the classical or alternative pathway C3 convertases (16). Data presented here suggest that heparin interferes with the generation of C3b on IC rather than blocking the binding of IC-C3b to erythrocyte CR_1. However, it is possible that heparin could interfere with the binding of IC to erythrocytes by other mechanisms as well. CVF appears to act as an unusually stable C3b,Bb (C3 convertase) which results in depletion of complement components by uncontrolled activation of the alternative pathway (17). Thus, both heparin and CVF interfere with the generation of C3b sites on IC. This in turn prevents binding of IC to CR_1.

CR_1 are expressed on the membranes of many cell types including macrophages, polymorphonuclear leukocytes, and primate erythrocytes (18, 19). However, data presented here indicate that, in vivo, blood leukocytes bind only minimal

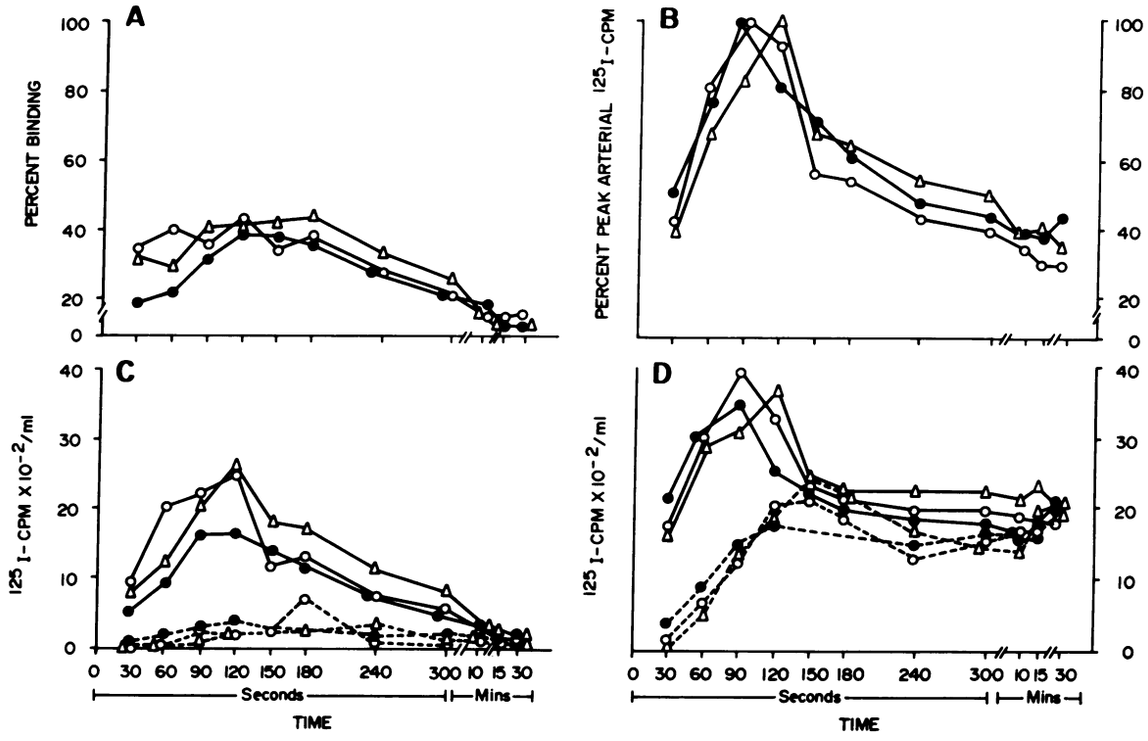

Figure 7. Effect of repeated IC infusions in a normal baboon. A dose (0.91 mg) of IC slightly greater than used in the complement-D studies was infused over a 2-min period. Periodic blood samples were obtained from the arterial circulation and hepatic vein. At 30 min, the IC clearance experiment was repeated. Finally, 30 min after the commencement of the second IC infusion, the experiment was repeated a third time using the same dose of IC. The data obtained from the first IC infusion (○), the second IC infusion (●), and the third IC infusion (△) are expressed as the percent binding of IC to erythrocytes (*A*), the percent peak arterial ^{125}I cpm (*B*), the erythrocyte-bound ^{125}I cpm in the arterial circulation (——) and hepatic vein (- - -) (*C*), and the ^{125}I cpm not bound to erythrocytes in the arterial circulation (——) and hepatic vein (- - -) (*D*).

amounts of IC relative to the quantity of IC bound by erythrocytes. These findings are similar to the studies of Medof and Oger (20) which examine the IC binding capacity of blood cells in vitro.

In the complement-D state, IC binding to both erythrocytes and leukocytes was markedly depressed. These data indicate that IC binding by erythrocytes in vivo is complement-dependent, just as it is in vitro (5, 20). The fact that blood leukocytes do not show a compensatory increase in IC binding under conditions in which IC are prevented from binding to erythrocytes because of complement-D, suggests that Fc receptor-mediated binding of IC to leukocytes is quantitatively a minor event.

The hepatic uptake of IC was found to be highly efficient under conditions of complement-D. This suggests that hepatic macrophage IC uptake is largely Fc receptor mediated. It is possible, however, that the affinity of hepatic macrophage CR_1 is sufficiently great that the low numbers of C3b sites on IC during complement-D were not rate limiting for efficient binding. Local synthesis of complement components in the liver of CVF- or heparin-treated primates, sufficient to permit binding of IC to hepatic CR_1 receptors, also cannot be excluded.

Perhaps the most interesting observation presented here is the accelerated clearance of IC from the circulation of complement-D primates. Differences in the rate of IC clearance in complement-D vs. complement-N primates cannot be attributed to the sequence in which the animals received the two IC challenges, since IC clearance under normal conditions was measured first in heparin-treated primates, whereas the sequence (complement-D vs. complement-N) was reversed in the CVF experiments. The difference in IC clearance under complement-D vs. complement-N also is unlikely to be explained as the basis of differences in hemodynamics between the two states. Heparin infusion had no apparent effect on hemodynamics as assessed by blood pressure, pulse rate, and general conditions of the animal and, although plasma infusion in the CVF experiments resulted in moderate volume expansion, the presumed increase in cardiac output should have lead to an increase in splanchnic blood flow. The resulting increase in hepatic and splenic blood flow should have favored increased

Table VI. Absolute Hepatic, Nonhepatic and Total IC Uptake from Blood during the Period of Rapid Clearance in Complement-N Vs. Complement-D

Experiment number	Total IC uptake*	Hepatic IC uptake*	Nonhepatic IC uptake*
	cpm × 10⁻⁵	*cpm × 10⁻⁵*	*cpm × 10⁻⁵*
Complement-N			
CVF #1	2.68	2.05	0.63
CVF #2	−3.41	2.39	−5.8
CVF #3	3.54	—	—
Heparin #4	4.77	1.87	2.90
Heparin #5	2.94	0.42	2.52
Heparin #6	2.36	0.87	1.49
Mean	2.15±1.2	1.52±0.4	0.35±1.6
Complement-D			
CVF #1	29.9	2.85	27.0
CVF #2	79.8	3.62	76.2
CVF #3	7.7	—	—
Heparin #4	13.4	1.93	11.5
Heparin #5	8.5	2.25	6.75
Heparin #6	1.6	0.48	1.12
Mean	23.5±11.9	2.23±0.5	24.4±13.7

* As defined in Methods.

hepatic and splenic IC uptake and an increased rate of IC clearance from the blood. Thus, in the CVF experiments, it is all the more remarkable that under conditions of complement-N (post plasma infusion), IC clearance from the blood was actually slower than under conditions of complement-D (pre-plasma infusion).

Table VII. Fractional Hepatic IC Uptake from the Erythrocyte Fraction and From the Plasma/Buffy Coat Fraction of Blood during the Period of Rapid Clearance in Complement-N Vs. Complement-D

	Complement-N		Complement-D	
Experiment number	Erythrocyte fraction	Plasma/ buffy coat fraction	Erythrocyte fraction	Plasma/ buffy coat fraction
Fractional hepatic IC uptake*				
CVF #1	0.53	0.19	0.74	0.53
CVF #2	0.37	0.10	−0.17	0.39
Heparin #4	0.76	0.18	0.66	0.54
Heparin #5	0.70	0.43	0.96	0.94
Heparin #6	0.89	0.24	0.78	0.36
Mean	0.65±0.09	0.23±0.06	0.59±0.2	0.55±0.1‡

* As defined in Methods.

‡ $P < 0.01$, compared with fractional hepatic IC uptake from the plasma/buffy coat fraction under conditions of complement-N.

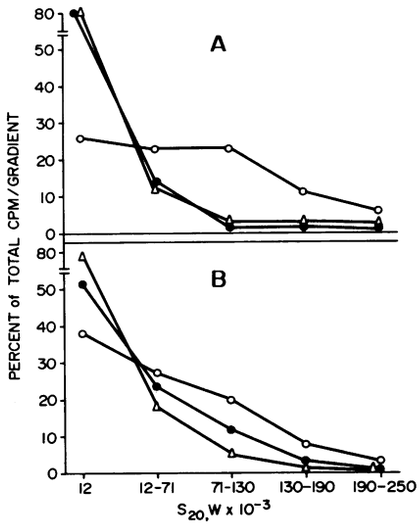

Figure 8. The size of IC remaining in the plasma after peak clearance of IC. Data from an experiment (heparin #6) in which complement was inhibited by heparin is shown in *A* (experimental protocol described in the legend of Fig. 2). The data for an experiment in which complement was depleted by CVF (CVF #3) is shown in *B* (experimental protocol described in the legend of Fig. 1). Blood samples, obtained at 180 s after the commencement of IC infusion, were centrifuged on isokinetic sucrose gradients. The data are expressed on the percentage of the total ^{125}I cpm per gradient in each fraction (expressed as S_{20}, W) for IC before infusion (○), IC obtained from baboons in the complement-depleted state (●), and IC obtained from normal (*A*) or plasma-reinfused (*B*) baboons (△).

Also to be considered is the possibility that under conditions of complement-N, the well demonstrated effect of C3b to intercalate itself in the IC lattice, which causes the degradation of the IC (10–13), may have occurred. This would result in persistence of smaller IC in the circulation of the complement-N animals. Thus, compared with the complement-D state, clearance rate of IC from the blood would be slower in the complement-N state because small IC persist in the circulation longer than large IC (21). Indeed, some degradation of IC may have occurred under conditions of complement-N (see Fig. 8 *B*). Note, however, that the higher percentage of smaller IC in the plasma in the complement-N vs. the complement-D state can be largely explained by the higher percentage of IC bound to erythrocytes in the complement-N state. That is, the larger IC in blood will preferentially bind to erythrocytes (5) and leave disproportionately greater amounts of small IC in plasma. However, even if degradation of IC by complement did occur it seems unlikely that degradation of IC by complement can alone account for the more rapid clearance of IC under conditions of complement-D, since: (*a*) The speed at which the IC are removed from the circulation in the present experiments would seem to be far too great to permit large scale modification of IC by complement-mediated degradation

(10–13). (*b*) If complement-mediated degradation were a major factor in the removal rate of IC from the circulation, the effect of complement-D on IC clearance should be demonstrable in nonprimates, which lack erythrocyte CR_1. However, in nonprimates, complement-D was not found to affect the sites of IC uptake or the rate of IC uptake by liver (1–3, 22–29). It should be noted, however, that the IC preparation used in the experiments in nonprimates were smaller than the IC used in the present experiments.

A reasonable hypothesis to explain the greater rate of IC clearance from the blood in the complement-D state is that the IC are free in the plasma rather than bound to erythrocytes and, thereby, are more susceptible to trapping by tissues. Thus, any condition that favors the presence of IC free in the plasma, rather than bound to erythrocytes, should favor accelerated clearance of IC from the circulation. In support of this hypothesis is our accumulated experience in assessing the factors modulating the erythrocyte-IC clearing mechanism. For example, in controlled studies in the primate it has been consistently demonstrated that IC that bind poorly to erythrocytes or are prevented from binding to erythrocytes are cleared more quickly from the circulation. This occurs even when the IC that bind less well to erythrocytes are much smaller than the IC which bind well to erythrocytes (14, 27).

Also in support of the hypothesis that IC in plasma are more susceptible to the IC uptake than IC bound to erythrocytes, are the findings of the present study that absolute hepatic IC uptake tends to increase during complement-D due to a striking increase in the fractional IC uptake out of the plasma fraction of blood. A similar phenomenon was noted for spleen plus gut. Although increased fractional IC uptake by kidney under conditions of complement-D was not detected, it should be noted that the method of measuring fractional IC uptake by assessment of arterial-venous differences across an organ makes it difficult to measure changes in fractional uptake by organs such as kidney, which have an intrinsic low fractional IC uptake. Liver has a fractional IC uptake nine times higher than that of kidney (6). Thus, differences in hepatic fractional IC uptake can be more readily identified by measuring arteriovenous differences. It should also be noted that only very small quantities of IC deposited in glomeruli are necessary to incite glomerulonephritis (28). Thus, even small increases in glomerular IC uptake, too small to be measured by the present techniques, could be critically important in the development of glomerulonephritis.

The nonhepatic sites of increased IC uptake, other than spleen plus gut, were not assessed in this study. However, if under the conditions of complement-D, IC uptake were distributed in proportion to the intrinsic capacity of tissues to trap IC, the other major sites of nonhepatic IC uptake would be skin, fat, lung, and skeletal muscles (6). It seems reasonable to assume that nonhepatic, nonsplenic deposition of pathogenic IC is potentially harmful.

The present study demonstrates that complement-D accelerates the removal of IC from the circulation and increases systemic (nonhepatic, nonsplenic) uptake of IC. The effects of complement-D appear to be mediated largely by interfering with the operation of the erythrocyte-IC clearing mechanism. The complement system could also affect IC clearance rates by increasing IC degradation rate or by promoting inhibition of IC formation (10–13). Indeed, it seems likely that several mechanisms that involve the complement system work in concert to protect the body against IC-mediated disease. For example, it may be that circulating IC that are not removed promptly by the erythrocyte-IC clearing mechanism may be "processed" and stripped from the erythrocyte and then degraded by complement (29, 30). Thus, hypocomplementemic patients may be unable to adequately defend themselves against IC formation. This, in turn, could lead to or exacerbate IC-mediated diseases. Indeed, failure of the normal operation of the mechanisms described above may explain the paradox of the predisposition of patients with congenital complement deficiency to IC-mediated diseases (13, 31–35), the apparent greater likelihood of relapse of systemic lupus erythematosus in patients who are persistently hypocomplementemic (36–38), and the association of IC-mediated glomerulonephritis in patients who are hypocomplementemic due to the activity of C3 nephritic factor (39, 40). These observations suggest that complement levels and the erythrocyte CR_1 receptor in primates may be important factors in the defense against IC-mediated diseases.

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