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Clearance and tissue uptake of immune complexes in complement-depleted and control mice

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Summary. The clearance kinetics, specific hepatic uptake and specific splenic uptake of immune complexes were examined in control mice and in mice treated with large doses of purified cobra venom factor (CoF) to deplete serum C3. At least 90% depletion of C3 was achieved as tested by double diffusion with antiserum specific to antigenic determinants on C3. A saturating dose of preformed immune complexes, consisting of HSA and rabbit antibodies to HSA, was used in these experiments. No differences in clearance kinetics and organ uptake of the immune complexes containing IgG as antibodies were observed between the two groups of mice. Within the limits of the experimental system no evidence was obtained for the participation of serum C3 and C3b receptors on Kupffer cells in the hepatic uptake of circulating immune complexes. The apparent discrepancies on the role of C3 and C3b receptors between these experiments and the *in vitro* studies on the uptake of immune complexes by macrophages is most likely related to the differences in the lattice of immune complexes employed by investigators.

INTRODUCTION

Immune complexes are involved in the pathogenesis of many disorders. The mononuclear phagocyte system constitutes a protective mechanism by removing circulating immune complexes. In experimental animals, preformed, large-latticed immune complexes, defined as containing more than two antibody molecules, were rapidly removed by the Kupffer cells of the liver (Arend & Mannik, 1971; Mannik & Arend, 1971; Haakenstad & Mannik, 1974). The efficient handling of these complexes by the cells of the mononuclear phagocyte system decreased their deposition in other tissue sites such as renal glomeruli (Haakenstad, Striker & Mannik, 1976). Both Fc and C3 receptors have been documented on Kupffer cells that originate from bone-marrow-derived monocytes (Crofton, Diesselhoff-den Dulk & Van Furth, 1978). The relative role of these two sets of receptors in removal of circulating soluble immune complexes, however, has remained controversial on the basis of *in vivo* and *in vitro* investigations.

Clearance and hepatic uptake of small doses of preformed, soluble immune complexes in rabbits were not altered by depletion of complement components with CoF or with aggregated IgG (Arend & Mannik, 1971; Mannik, Arend, Hall & Gilliland, 1971). Furthermore, the *in vitro* attachment of similar preparations of immune complexes to rabbit macrophages in suspension was comparable in the presence of fresh serum and in the presence of decomplemented serum (Arend & Mannik, 1972). On the other hand, the phagocytosis of immune complexes, containing larger than 30S material, by adherent mouse peritoneal macrophages

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was facilitated by complement (Van Snick & Masson, 1978). Similarly, the presence of complement enhanced the phagocytosis of heat-aggregated IgG by guinea-pig peritoneal macrophages, provided the number of IgG molecules per aggregate exceeded 16 (Kijlstra, vanEs & Daha, 1979). The latter observations suggest that C3 and C3 receptors are important in handling of immune complexes by macrophages and Kupffer cells.

For these reasons, the present study examined the clearance kinetics and hepatic uptake of saturating doses of soluble immune complexes in CoF-treated and control mice to determine the role of serum C3 and thereby indirectly the role of C3 receptors in the clearance of circulating immune complexes.

MATERIALS AND METHODS

Preparation of antigens, antibodies and soluble immune complexes

The used procedures were detailed previously (Mannik *et al.*, 1971; Haakenstad & Mannik, 1974) and are only summarized here. Antibodies to HSA (anti-HSA) were isolated from hyperimmune rabbit serum by immunoadsorbents and were trace-labelled with ^{125}I. Monomeric antibodies and monomeric HSA were obtained by gel filtration in borate buffered saline (0·2 M borate, 0·15 M NaCl, pH 8·0). The protein concentrations were determined by absorbance at 280 nm using extinction coefficients of 6·6 for HSA and 14·4 for anti-HSA. The point of equivalence was determined by quantitative precipitin curves, and the complexes were prepared at five-fold antigen excess by adding antibodies to antigen with constant mixing. The preparations were centrifuged at 1000 ***g*** for 20 min prior to use. The molar composition and sedimentation characteristics of similar HSA and anti-HSA complexes have been described (Mannik *et al.*, 1971; Haakenstad & Mannik 1974; Arend, Teller & Mannik, 1972). The prepared complexes had no significant radioactivity in the pellet upon ultracentrifugation in 10%–30% sucrose density gradients under previously used conditions (Haakenstad & Mannik, 1974). Normal rabbit IgG was labelled with ^{131}I, rendered monomeric by gel filtration and used as a blood volume marker as previously described (Arend & Mannik, 1971).

Disappearance of immune complexes from the circulation

C57Bl/6J female mice, weighing 18–22 gm (Jackson Laboratories, Bar Harbor, Maine), received a single 0·5 ml tail-vein injection of complexes containing 5 mg of anti-HSA, a dose that showed saturation of the hepatic uptake of complexes in a previous study (Haakenstad & Mannik, 1974). The animals received potassium iodide in their drinking water at least 24 hr prior to injection and during the experiment.

The methods for assessing the disappearance of radioactivity from the circulation have been previously described (Mannik *et al.*, 1971; Haakenstad & Mannik, 1976). Briefly, 10 μl blood samples were obtained from the retro-orbital plexus at 5, 10, 15 and 30 min and 1, 2, 4, 8, 12, 24, 48, 72 and 96 hr following an injection. The radioactivity in the sample was determined in an automatic well-type gamma counter. The fraction of radioactivity in the blood sample of each mouse was calculated relative to a zero time value, which was determined by extrapolation of the log counts per min at 5, 10 and 15 min. The mean and standard deviations of the values for three or four mice receiving the preparation were calculated at each sampling time. A previously described analysis (Mannik *et al.*, 1971) of these data by the SAAM-27 programme (Berman & Weiss, 1978) computed a curve for the disappearance of each preparation from the circulation as well as the number of exponential components composing the curve, the half-life of each component and the proportion of the injected preparation cleared at that half-life.

Specific organ localization of immune complexes

The specific liver and spleen localization of the same immune complexes was determined in mice killed at 1, 4, 12, 16, 24 and 36 hr after injection of complexes. For this purpose, mice received an injection of 200 μg of normal rabbit ^{131}I-IgG 5–10 min prior to death as blood volume marker. The ^{125}I and ^{131}I counts per min in the organ were determined and the ^{125}I specific uptake was calculated as follows:

$$^{125}\text{I specific uptake} = \frac{^{125}\text{I c.p.m.}}{\text{organ}} - \frac{^{131}\text{I c.p.m./organ}}{^{131}\text{I c.p.m./ml blood}} \times {}^{125}\text{I c.p.m./ml of blood}$$

Depletion of complement

Lyophilized venom from the cobra *Naja Naja* was obtained from the Miami Serpentarium (Miami, Florida). Purified low molecular weight anti-complementary factor (CoF) was obtained by the method described by Cochrane, Müller-Eberhard & Aikin (1970)

utilizing DEAE-cellulose and Sephadex G 200 chromatography. In addition, the phospholipase A_2 activity in the CoF was removed with p-bromophenacyl bromide according to the method of Shaw, Roberts, Ulevitch, Henson & Dennis (1978).

The units of purified CoF were determined by described methods (Cochrane *et al.*, 1970). The mice used for study of disappearance kinetics received 70 u of CoF intraperitoneally by the following schedule: four injections of 10 u each, beginning 24 hr before the administration of complexes and spaced at about 6 hr intervals, followed by injections of 10 u 24, 48 and 72 hr after the administration of complexes. The mice used for organ uptake studies received 60 u of CoF intraperitoneally, 20 u were given three times during the 24-hr period before injection of complexes. Further CoF was not injected because these mice were killed 1–36 hr after the administration of complexes. The CoF dose used in this study was considerably higher than used in mice by other investigators (Simpson, Moran, Evans & Peters, 1978; Rumjanek, Brent & Pepys, 1978). No adverse effects from CoF were observed in the treated mice.

Blood samples for determination of complement levels were drawn before the decomplementation treatment, and 48 hr and 96 hr after administration of complexes. Serum was immediately separated and frozen at $-70°$ for later studies. The serum levels of C3 were estimated from the greatest serum dilution at which a precipitin line was detected by double diffusion, using a rabbit antiserum specific to mouse C3 prepared in rabbits by the method of Mardinay & Müller-Eberhard (1964). This antiserum reacted with mouse C3, C3b, and C3d by immunoelectrophoresis and by double diffusion.

Immunofluorescence studies of liver and kidney

Kidney and liver sections were removed after an anesthetized mouse was killed by exsanguination and immediately embedded and frozen in Tissue-Tek II O.C.T. compound (Ames Company, Elkhart, Indiana) and stored at $-70°$ for immunofluorescence microscopy.

Five microgram sections were stained with fluorescein-conjugated goat anti-rabbit IgG, which had been checked for specificity on agarose-antigen beads (Case, Lussier & Mannik, 1975), and with fluorescein-conjugated goat anti-mouse C3 (Cappel Laboratories, Cochranville, Pennysylvania). The specificity of the anti-C3 by immunoelectrophoresis was directed to C3. The sections were examined with a Zeiss Universal epi-fluorescence microscope with previously described optics (Haakenstad *et al.*, 1976). Sections were graded 0–4+ for intensity. The observer was not aware of the number of the animal or the group or time to which the animal belonged. The sections were photographed with Tri-X pan ASA 400 film (Eastman Kodak, Rochester, New York).

RESULTS

To determine whether the presence of serum C3 and, therefore indirectly, C3 receptors are involved in the clearance of circulating immune complexes, clearance kinetics were examined over a 96-hr period in complement-depleted and control mice. For this purpose three control and four CoF-treated mice were studied. C3 was detected at 1:32 or 1:64 dilutions of serum before CoF treatment and in control mice. After CoF treatment C3 antigenicity was detected only in undiluted or in 1:2 dilutions of serum, representing either intact C3 or breakdown products thereof. Thus, at least 90% depletion of C3 was achieved. In C3-depleted and in control mice the disappearance of injected immune complexes consisted of three exponential components (Fig. 1 and Table 1). No significant differences were found in the disappearance from the circulation in the two groups at any time-point. In particu-

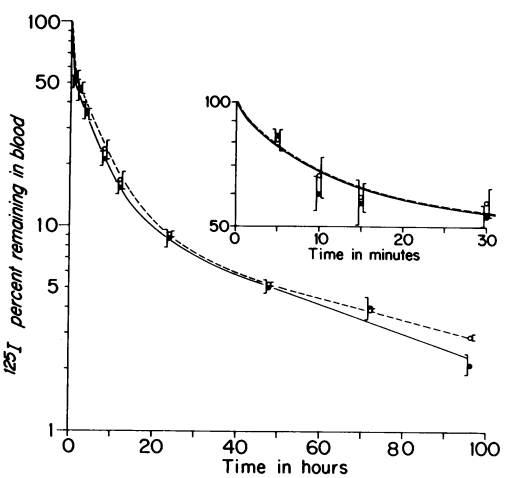

Figure 1. Computer-fitted curves for the disappearance of HSA-anti-HSA complexes in control and CoF-treated mice. The curves were fitted to the mean ± 1SD for three mice in the control group (o– – –o) and for four mice in the CoF-treated group (●——●). The insert indicates the initial phase of disappearance during the first 30 min. No significant differences existed at any time-points between the two groups.

Table 1. Calculated exponential components describing the disappearance of circulating immune complexes in control and CoF-treated mice

	First component		Second component		Third component	
	t 1/2 (hr)	%	t 1/2 (hr)	%	t 1/2 (hr)	%
Control N = 3	0·086 ± 0·014	41·97 ± 1·54	5·17 ± 0·22	48·88 ± 1·37	58·24 ± 2·55	9·18 ± 0·34
CoF N = 4	0·095 ± 0·016	43·26 ± 2·65	4·06 ± 0·46	44·97 ± 2·65	40·29 ± 4·70	11·87 ± 1·48

lar, no differences existed in the individual time-points in the first 24 hr that account for the removal of the bulk of the large-latticed immune complexes by the liver. This fact was also expressed by the similarity of the calculated half-lives and percentages of the first two exponential components of these curves (Table 1). The half-lives of these components were similar to previously published data (Haakenstad & Mannik, 1976). The third exponential component had a half-life of 40·29 ± 4·70 hr in treated mice and 58·24 ± 2·55 hr in control mice, but the third exponential component represents the clearance of small-latticed complexes that were not rapidly taken up by the liver (Haakenstad & Mannik, 1976) and not deposited in glomeruli (Mannik & Striker, 1980).

Quantitative tissue uptake

To further confirm that the CoF treatment did not alter the hepatic uptake of large-latticed immune complexes, the specific hepatic and splenic uptake of these materials were examined quantitatively. For example, one could argue that although the clearance kinetics are identical, this could result from a decreased hepatic uptake and a compensated increase in splenic uptake of immune complexes, thereby producing identical clearance curves. This was not the case.

The specific hepatic uptake of immune complexes was examined in complement depleted and control mice at 1, 4, 12, 16, 24 and 36 hr. No statistical difference at any time-point between the two groups was observed (Fig. 2). During the first 4 hr an increasing hepatic uptake of immune complexes was observed in both treated and control groups. The highest quantity of radioactivity in the liver was found during the time-interval from 4 to 16 hr. From 16 hr on the total

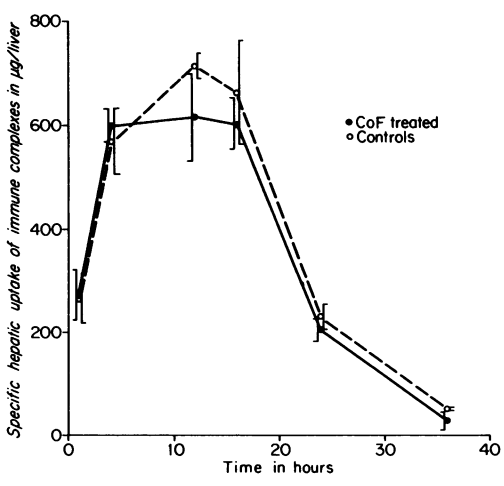

Figure 2. Specific hepatic localization of immune complexes in control and CoF-treated mice. The mean ±1 SD are indicated for each time-point for three control and for three CoF-treated mice. No significant differences existed at any time-point between the two groups.

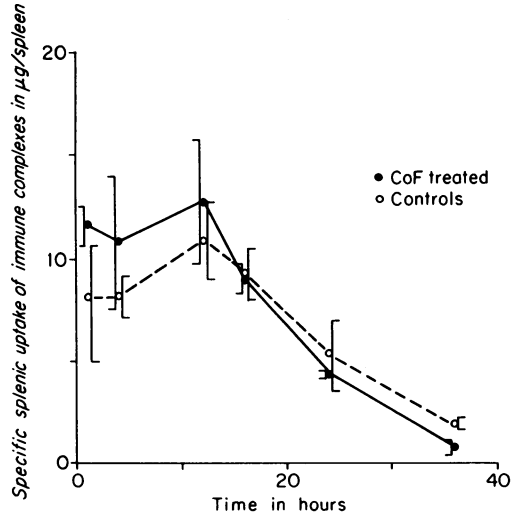

Figure 3. Specific splenic localization of immune complexes in control and CoF-treated mice. The mean ±1 SD are indicated for each time-point for three control and three CoF-treated mice. No significant differences existed at any time-point between the two groups.

amount of complexes in the liver declined, reflecting a degradative function. This catabolic phase, like the earlier phase of phagocytosis, was identical in both groups. Similar studies were carried out with splenic uptake of immune complexes at 1, 4, 12, 16, 24 and 36 hr (Fig. 3). The maximum uptake, like in the liver, occurred at 12 hr. The maximum uptake was only 12 μg/spleen, indicating a very limited uptake of soluble complexes by the spleen. The maximum presence of complexes per gram of liver and spleen were 711 and 234, respectively, in the CoF-treated and 738 and 194 in the controls, still indicating the preferential uptake of soluble complexes by the liver, when the uptake was calculated per gram of tissue.

Immunofluorescence microscopy

Sections of liver were examined by immunofluorescence microscopy for rabbit IgG, reflecting the presence of immune complexes, and for mouse C3 in the same control and experimental mice used for quantitative uptake of complexes. The presence of rabbit IgG paralleled the quantitative analysis, reaching 4+ at four hr and declining after 16 hr. C3, however, was not detected by immunofluorescence microscopy in the Kupffer cells of control and C3-depleted mice at any time-period (see Fig. 4, for example).

Kidney sections were stained similarly for rabbit IgG and for mouse C3 in both complement-depleted and control mice. Table 2 depicts the intensity of immunofluorescent staining in kidney sections at 1, 4, 12, 16, 24 and 36 hr. Glomerular staining for C3 was not detected at any time-point in the kidney sections of CoF-treated mice (see Fig. 5). On the other hand, C3 was detected in the control mice after the first hour. These data further show that C3 was depleted to a level that precluded glomerular deposition of this protein to a detectable degree.

Table 2. Intensity (*) of glomerular immunofluorescence in mouse kidneys after staining for rabbit IgG and for mouse C3

	Control mice		CoF-treated mice	
Time (hr)	Rabbit IgG	Mouse C3	Rabbit IgG	Mouse C3
1(†)	1+	0	1–2+	0
4	1–2+	1–2+	1–2+	0
12	1–2+	tr–1+	1–2+	0
16	2+	tr–1+	2+	0
24	1–2+	0–tr	1–2+	0
36	1+	0–tr	1–2+	0

(*) The intensity of fluorescence was graded from negative (0) to 4+; tr indicates trace amounts.

(†) At each time-point two or three mice were examined and the range of intensity of staining is indicated, if variations occurred between mice

DISCUSSION

In systemic immune complex disease, such as systemic lupus erythematosus, the concentration of serum complement and complement components, including C3, tend to be below normal range during active disease (Williams & Law, 1958; Kohler & Ten Bensel, 1969; Ruddy, Everson, Schur & Austen, 1971). If serum C3 and C3 receptors of Kupffer cells had an important role in the clearing of circulating immune complexes by the mononuclear phagocyte system, then lowering of the serum C3 level might further decrease removal

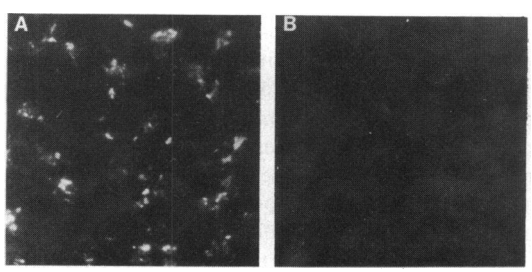

Figure 4. Representative liver sections from a control mouse (4 hr), stained with fluorescein-conjugated antibodies to rabbit IgG in (A) and to mouse C3 in (B). Even though abundant immune complexes were present in Kupffer cells, these complexes did not stain for C3 as shown in (B).

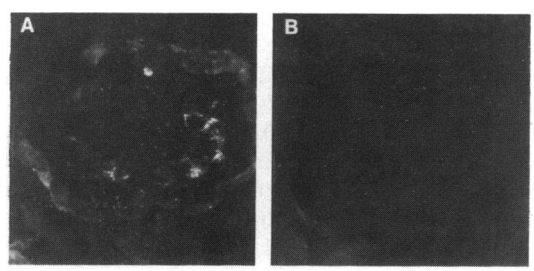

Figure 5. Representative glomeruli in the CoF-treated and control mice (12 hr), stained with a fluorescein-conjugated antiserum to mouse C3. In the control section (A), C3 is stained in the glomerulus in a mesangial pattern, but in the CoF-treated mouse (B) even though immune complexes were present, no staining for C3 is observed. This antiserum always stained Bowman's capsule for reasons that were not elucidated.

of circulating complexes and thereby enhance tissue deposition of complexes, which in turn would increase organ damage.

Studies in experimental animals, however, have indicated that a substantial decrease in serum C3 does not alter the clearance kinetics and specific hepatic uptake of immune complexes containing IgG as antibodies. When rabbits were given small doses of preformed immune complexes, the clearance kinetics (Mannik *et al.*, 1971) and the specific hepatic uptake (Arend & Mannik, 1971) were comparable in control rabbits and in rabbits treated with CoF or with aggregated human IgG, to deplete C3 or early complement components respectively. In the present study with saturating doses of preformed immune complexes, again the clearance kinetics of circulating complexes and the specific hepatic and specific splenic uptake were not different in control mice and in mice treated with high doses of CoF. In all of these cited studies comparable preformed immune complexes were injected that included about 50% of the antibody in complexes exceeding the lattice of Ag_2Ab_2, that ranged in sedimentation coefficients from about 14S to a very small proportion at 22S, as calculated from linear sucrose density gradient ultracentrifugation (Mannik *et al.*, 1971). Furthermore, comparable immune complexes *in vitro* showed no differences in adherence to rabbit macrophages in suspension cultures in the presence of fresh or heat-decomplemented serum (Arend & Mannik, 1972).

In contrast, other *in vitro* studies have clearly demonstrated that the presence of complement in rather low concentrations facilitates the phagocytosis of immune complexes or stable, heat-aggregated IgG by macrophages, but larger immune complexes or aggregates were used than in the experiments described herein. Van Snick & Masson (1978) employed immune complexes of human transferrin and mouse antibodies to this protein to demonstrate the enhanced phagocytosis and degradation of immune complexes in the presence of complement. In their experiments immune complexes greater than 30S were present. The proportion of these very large complexes decreased at ten-fold antigen excess and the rate of uptake by macrophages declined, but was still facilitated by presence of complement. The nature of complexes that were actually taken up by the adherent macrophages was not characterized. The significant decline in the uptake of immune complexes made at ten-fold transferrin excess, however, suggests that complexes sedimenting faster than 30S were preferentially phagocytozed by the macrophages and that only the uptake of these complexes was facilitated by complement. In addition, the studies by Kijlstra *et al.* (1979) showed that the phagocytosis and degradation of aggregated IgG by adherent guinea-pig macrophages was enhanced by complement only when the number of IgG molecules in the aggregates exceeded sixteen. In fact, these investigators provided some evidence that phagocytosis of aggregates composed of ten IgG molecules was inhibited by fresh serum in comparison to medium alone. In the same study Kijlstra *et al.* (1979) showed that with increasing number of IgG molecules the aggregates became more effective in the activation of the complement cascade, as measured by consumption of the limited amount of C4 in the system. From these considerations it is apparent that the lattice of immune complexes or the number of IgG molecules in aggregates are important in determining if C3 and C3 receptors facilitate the uptake of immune complexes by macrophages in culture systems. Presumably, these large complexes or aggregates actually bind C3b, thus enhancing their uptake by macrophages. The experiments described in this report and previous investigations by Arend & Mannik (1971) and Mannik *et al.* (1971) employed immune complexes that did not reach sedimention characteristics of the materials used by Van Snick & Masson (1978) and by Kijlstra *et al.* (1979). These differences in experimental design may well explain the difference in the conclusions reached in regard to the role of C3 receptors on Kupffer cells and macrophages in phagocytosis of immune complexes. We have purposefully not employed immune complexes with very high degrees of lattice formation since these become entrapped in pulmonary circulation upon intravenous injection. The mechanisms for this entrapment have not been examined in detail.

The immune complexes used in the present study were cleared from the circulation due to interaction with Fc receptors on Kupffer cells without any contribution from the interaction of C3 and C3 receptors on these cells. This conclusion was based on the findings that at least 90% depletion of serum C3 by CoF did not alter the clearance kinetics of the injected complexes and did not decrease the specific hepatic uptake of the complexes. The absence of involvement of C3 and C3 receptors on Kupffer cells in the uptake of the injected complexes was further suggested by the failure to find C3 in Kupffer cells of control animals by immunofluorescence microscopy. The finding of C3 in glomerular deposits of control mice by the same technique provided some confidence in the used immuno-

fluorescence method. Of note is the fact that the deposition of immune complexes containing rabbit IgG preceded the appearance of C3 in the deposits in this study and in earlier experiments (Haakenstad *et al.*, 1976), indicating that complexes were deposited in glomeruli prior to binding C3 components and that binding of C3 occurred after the deposits in glomeruli had reached a critical size or mass. These observations are all consistent with the notion that the uptake of complexes by the Kupffer cells in the present study was mediated by the Fc receptors and not facilitated by C3b receptors, most likely due to the failure of these complexes to bind C3b. Mannik *et al.* (1971) and Arend & Mannik (1972) showed that HSA and anti-HSA complexes, comparable to the complexes used in this study, depleted total haemolytic complement as measured by inhibition of lysis of sensitized red cells. This complement fixation, however, may have reflected binding of C1q to the complexes and did not imply binding C3b to the complexes as was suggested by Kijlstra *et al.*(1979). The fate and uptake by Kupffer cells of circulating immune complexes with known amounts of bound C3b has not been determined.

The treatment of mice with CoF in this study resulted in at least 90% depletion of C3 as detected by double diffusion. The depletion of C3 was also supported by the fact that in treated mice C3 deposition was not found in renal glomeruli, whereas this protein accumulated in glomeruli of control mice. Nevertheless, it is important to keep in mind during the interpretation of presented data that the CoF depletes C3 by activation of this complement component and not by interfering with synthesis of this protein (Lachmann & Nicol, 1973; Alper & Balavitch, 1976). Therefore, C3 is continuously released from the cells synthesizing this protein and then inactivated by the CoF. Thus, if the studied biological activity required only small amounts of C3, then the depletion of C3 with CoF may not reveal any differences. Interestingly, the phagocytosis of large aggregates of IgG by macrophages in culture was facilitated by the addition of as little as 1% of fresh serum to the culture as a source of complement (Kijlstra *et al.*, 1979). Furthermore, C3 has been shown to be synthesized by macrophages as reviewed by Colten (1976).

Nevertheless, the presented data with all the discussed reservations indicate that a major decrease in serum C3 does not alter the clearance and hepatic uptake of circulating immune complexes containing IgG as antibodies. These observations at least imply that decreases of total complement and C3 seen in clinical immune complex diseases do not contribute to decreased removal of circulating immune complexes and prolonged circulation and enhanced tissue deposition of these substances. On the other hand, on the basis of reported *in vitro* studies, depressed C3 levels in immune complex diseases may influence the rate of removal of very large immune complexes that effectively bind C3b. Studies in experimental animals with such large complexes, however, have not been conducted.

Complement peptides C4a, C3a and C5a have been shown to have anaphylatoxic properties, including increase of vascular permeability (Gorski, Hugli & Müller-Eberhard, 1979). In the presented data the rapid removal of immune complexes during the first few minutes represented increased vascular permeability, as shown by studies of Haakenstad, Case & Mannik (1975). The CoF-treated and control mice had identical early phases of removal of complexes, suggesting that the C3 depletion did not alter this function or that peptides other than C3a mediate the increased vascular permeability.

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