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Delayed removal of renal-bound antigen in decomplemented rabbits with acute serum sickness

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SUMMARY

We have examined the effects of complement depletion, brought about by cobra venom factor (CVF), on the clearance of radiolabelled BSA from the kidney in acute serum sickness in rabbits. The decomplemented group showed a significant decrease in the rate of clearance of renal-bound antigen with a mean half-clearance time of 19·5 days (range 9·3–60) *vs* 11·3 (range 8·7–15) for the control group. C3 was rapidly cleared from the glomerulus (as assessed by immunofluorescence) when further deposition was prevented by treatment with CVF. Glomerular immune complexes apparently activated the alternative pathway *in vitro*. These findings suggest that in immune complex nephritis, the clearance of deposited complexes from the kidney is complement-dependent.

INTRODUCTION

The mechanisms of deposition of circulating immune complexes in the kidney have been extensively studied in experimental systems (Cochrane & Koffler, 1973), but little work has been undertaken on the local mechanisms involved in their removal. Studies to date (Valdes *et al.*, 1969; Wilson & Dixon, 1971) have shown that deposited complexes are in some kind of equilibrium with those circulating and, particularly, that the administration of massive doses of antigen (BSA) generating small and relatively highly soluble complexes also results in an accelerated removal of antigen from the kidney.

The dissolution of immune precipitates, which occurs when they are incubated with fresh normal serum, is dependent upon activation of the alternative pathway of complement (Miller & Nussenzweig 1975; Czop & Nussenzweig, 1976). This work, together with the observation that C3 is deposited in the glomerulus in increasing amounts during the healing phase of acute serum sickness (Fish *et al.*, 1966; Wilson & Dixon, 1970) suggested that complement-dependent dissolution of immune complexes might occur in the glomerulus. We have therefore investigated this possibility by examining the effect of administration of cobra venom factor in doses sufficient to induce systemic decomplementation on the clearance of BSA from the glomerulus in rabbits with BSA-induced serum sickness, and whether glomerular deposits of antigen–antibody complexes can activate the alternative pathway *in vitro*.

MATERIALS AND METHODS

Animals. New Zealand white rabbits weighing between 2·0 and 2·5 kg were used. The animals were fed on a pellet diet and their drinking water contained 0·02% NaCl and 0·04% potassium iodide.

Radiolabelled protein. Bovine serum albumin (BSA, Armour) and rabbit serum albumin (RSA) prepared from normal rabbit serum (Weigle, 1961) were labelled by the chloramine T method with ^{125}I (BSA) and ^{131}I (RSA) (McCohaney & Dixon, 1966).

CVF. CVF was purified from *Naja naja* venom (Sigma) by DEAE-cellulose and Sephadex G-200 chromatography (Ballow & Cochrane, 1969).

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C3 levels. C3 levels were measured in EDTA–plasma samples by rocket immunoelectrophoresis (Laurell, 1965) using a monospecific sheep anti-rabbit C3. The C3 level for each animal is expressed as a percentage of that found on day 4 before immunization.

Complement reagents. EDTA–guinea-pig serum (EDTA–GPS) contained 0·01 M EDTA. C4-deficient guinea-pig serum (C4-D GPS) was obtained from the strain of guinea-pigs genetically deficient in C4 (NIH). Rabbit serum was depleted of calcium by 0·01 M EGTA and 0·005 M magnesium (Mg–EGTA/RS). Rabbit serum depleted of functional factor B was prepared by heating serum at 50°C for 20 min (RB-RS).

Fluorescein-labelled antisera. Sheep anti-rabbit IgG was purchased from Wellcome Laboratories, UK. Rabbit antisera to BSA and guinea-pig C3, and goat antiserum to rabbit C3, were prepared and fluorescein-conjugated in our laboratory (Fothergill, 1969).

Induction of ASS. Twenty-six rabbits were pre-immunized with 3·0 mg of unlabelled BSA in Freund's complete adjuvant on day −4. On day 0, 250 mg/kg of ^{125}I-labelled BSA was administered intravenously. The rate of antigen elimination from the serum was measured by the determination of ^{125}I-labelled BSA in aliquots of EDTA–plasma precipitated with 10% trichloroacetic acid (TCA). Circulating immune complexes were assayed by the Farr technique, measuring radioactivity precipitated by 50% ammonium sulphate. Proteinuria was measured in catheter samples by the Biuret method.

Experimental protocol. Four animals which did not eliminate the antigen before day 15 were excluded, as delayed immune elimination results in little or no immune complex deposition within the kidney (Wilson & Dixon, 1970). The remaining twenty-two animals were matched according to the day on which 99% immune elimination occurred (day 99%) and divided into two groups: (a) the CVF-treated group (eleven rabbits) were given 70 units CVF per kg i.v. (dissolved in 10 ml of normal saline) over 5 to 10 min, 3 to 6 hr before nephrectomy; and (b) the control group (ten rabbits) (one died of unknown cause) were injected with 10 ml of normal saline alone.

^{131}I-labelled RSA was administered 6 to 12 hr before nephrectomy or autopsy.

Nephrectomy (specimen N) was carried out under neuroleptanalgesia (Hypnorm®, 0·5 ml/kg, i.m.),* using a clean but not sterile technique, 4 to 8 days after day 99%; the remaining kidney (specimen A) was removed 4 to 8 days later after a lethal dose of pentobarbitone i.v. All kidneys were stored at −70°C.

Calculation of quantities of BSA deposited and of the half-disappearance rate (t_{50}) of radiolabelled BSA from the kidneys. Renal-bound BSA was determined in Waring Blender kidney homogenates, as previously described (Bartolotti, 1977). The washed homogenates were precipitated by 20% TCA and counted. The amount of BSA found in each specimen (N or A) was multiplied by two and expressed as μg per total kidney weight (TKW). The t_{50} was calculated according to the following formula:

t_{50} (days) = 0·5 N/(N−A)d; where d = interval in days between nephrectomy and autopsy, N = nephrectomy counts and A = autopsy counts.

Histology and Immunofluorescence. Small pieces of kidney cortex were fixed in 10% buffered formalin, sectioned at 4 μm and stained with haematoxylin and eosin and PAS. Glomerular cellularity was estimated by counting the number of cells (polymorphonuclear (PMN) and non-polymorphonuclear cells (non-PMN) in ten representative glomeruli (of similar diameter) per section. Cryostat sections were examined using fluorescent antisera by techniques described previously (Evans *et al.*, 1973). The intensity and amount of immunofluorescence was arbitrarily graded from 0 to 3+, using a method similar to that described by Wilson & Dixon (1970); (0) negative: (trace) minimal glomerular staining; (1+) staining of most glomerular capillaries; (2+) staining of all glomeruli; and (3+) glomeruli filled with fluorescent deposits. The pattern of immunofluorescence were described as globular (large size) deposits or as a granular pattern (fine or coarse). Assessment was carried out in a 'blind' fashion.

In vitro complement fixation on kidney cryostat sections. In order to study whether glomerular immune precipitates are able to fix complement by the alternative pathway *in vitro,* the following experiments were carried out. Autopsy kidneys, from decomplemented animals (rabbits 18, 19 and 25) and from healthy control rabbits, were cut into 4 μm cryostat sections, air-dried for 20 min and washed in phosphate-buffered saline (PBS). Sections were then incubated at 37°C for 20 min with 20 μl of each of the following reagents: (i) EDTA–GPS; (ii) C4-DGPS; (iii) Mg–EGTA–RS; and (iv) RB-RS. The sections were then washed twice in PBS and stained with fluorescent antisera to rabbit and guinea-pig C3. Glomerular immunofluorescence was assessed as described earlier.

Statistical analysis. The Wilcoxon rank sum test for two samples (Wilcoxon, 1945) was used to compare the results in the two groups of animals.

RESULTS

General

The following values were not significantly different ($P > 0·1$) between the control and the decomplemented group.

(i) *Proteinuria.* The mean of the peak proteinuria before nephrectomy was 728 mg/100 ml (range: 207–2010) for the control group and 528 mg/100 ml (100–1500) in the CVF-treated group. Following

*Janssen Pharmaceutical, Beerse, Belgium.

TABLE 1. Renal-bound radiolabelled BSA in μg per total kidney weight (TKW) at nephrectomy and autopsy

Animals	Day 99%	Nephrectomy–autopsy interval (days)	BSA (μg)	
			Nephrectomy	Autopsy
Control group				
12	13	4	9·3	7·3
11	13	4	8·9	7·4
5	12	5	25·0	20·4
2	11	5	31·0	23·0
16	13	6	10·0	8·0
23	13	6	13·0	9·5
21	12	6	16·5	11·3
22	13	6	22·0	14·4
8	12	8	24·0	8·9
17	12	8	19·0	13·0
CVF group				
10	13	4	14·1	12·7
18	13	4	7·9	6·6
1	12	5	35·0	30·0
3	10	5	35·7	24·7
13	13	6	16·6	15·8
19	15	6	11·4	9·1
25	13	6	25·5	21·0
24	13	6	9·0	7·3
6	13	8	8·0	5·2
14	12	8	19·0	11·0
20	13	6	7·7	6·7

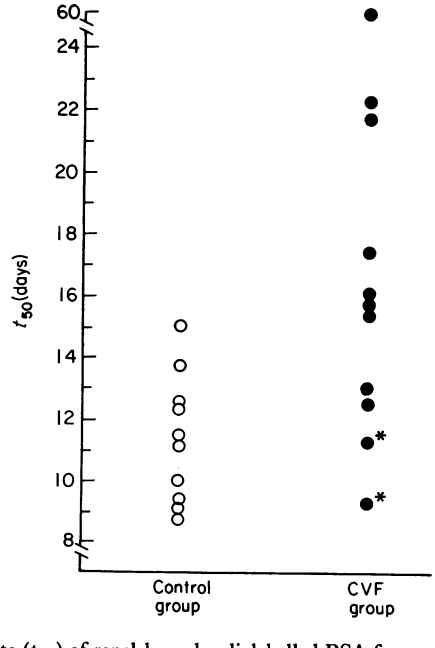

FIG. 1. Half-disappearance rate (t_{50}) of renal-bound radiolabelled BSA for control (○) and decomplemented (●) groups. The difference in t_{50} is statistically significant ($P < 0.01$) using the Wilcoxon rank sum test. *Rabbits with high plasma C3 levels.

TABLE 2. Glomerular cellularity (polymorphonuclear and non-polymorphonuclear cells) in the nephrectomy specimen and at autopsy

Non-PMN cell count		PMN cell count	
Nephrectomy	Autopsy	Nephrectomy	Autopsy
Control group			
53* (35–74)	51 (31–68)	1·5 (0·7–3)	2.7 (1·4–5·9)
CVF group			
56 (37–85)	49 (24–101)	4·3 (1·8–9·4)	1·55 (0·7–4·6)
$P > 0·1$	$> 0·1$	$< 0·01$	$> 0·1$

*Results are expressed as the mean cell count with the ranges in parentheses.

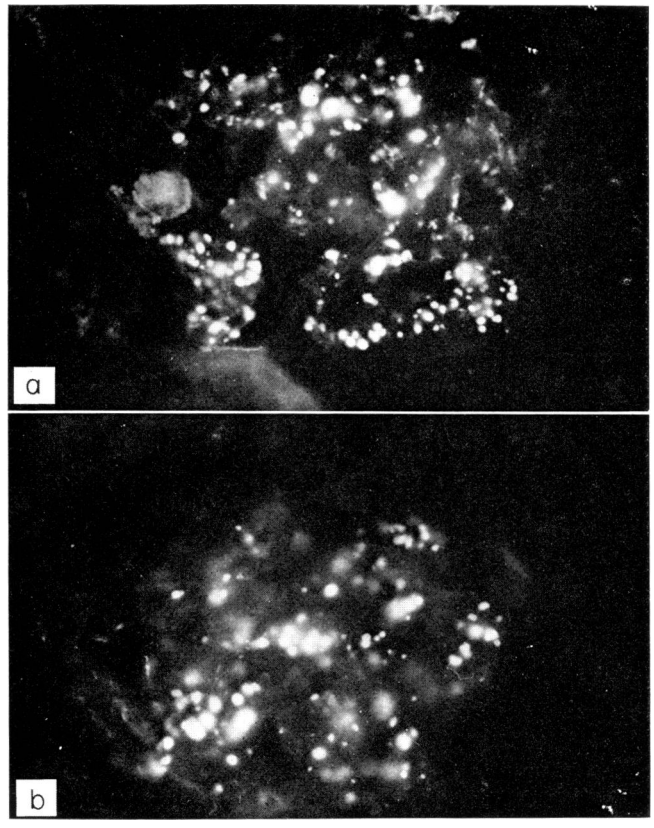

FIG. 2. Comparison of immunofluorescence (C3) in the nephrectomy (a) and autopsy (b) specimens from rabbit No. 2 (control). Note the similar pattern (globular) and intensity (3+) in both specimens. (Magnification × 500.)

nephrectomy, the peak proteinuria averaged 171 mg/100 ml (110–205) in the control group and 107 mg/100 ml (0–200) in the CVF-treated group.

(ii) *Immune complexes.* The maximum amount of ^{125}I-labelled BSA precipitable in 50% saturated ammonium sulphate averaged 2·1% (range: 1–3) of the total dose of BSA in the control group, and 2·2% (1·5–4) in the CVF-treated group.

(iii) *C3 levels (before CVF administration).* The lowest concentration of immunoreactive C3 (which

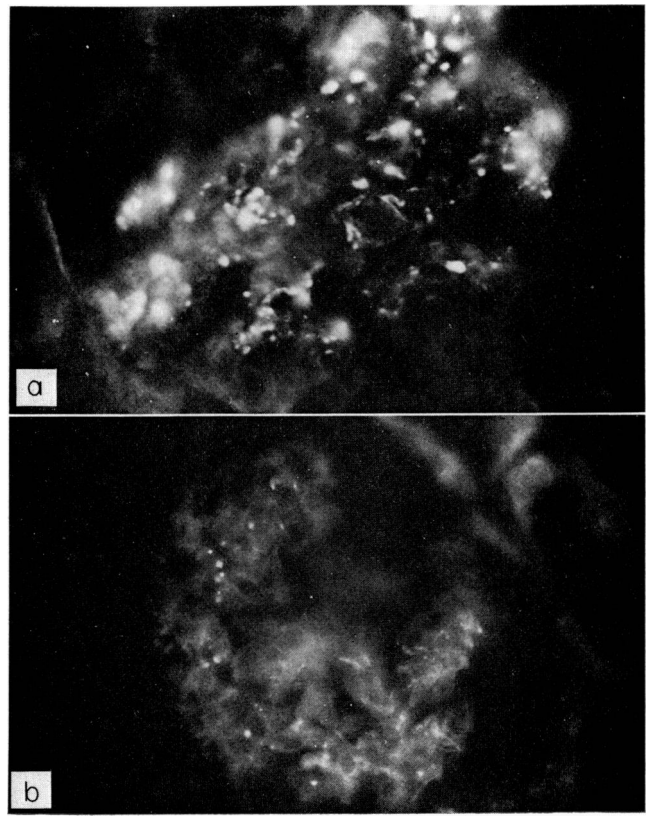

FIG. 3. Comparison of glomerular immunofluorescence (C3) in the nephrectomy (a) and autopsy (b) specimens in rabbit No. 20 (decomplemented). Note the globular pattern (2+) in the nephrectomy specimen but only trace deposits at autopsy. (Magnification × 320.)

occurred 1 day after 'day 99%' in eleven out of twenty-one animals) during the natural course of the disease averaged 50% (35–73) in the control group and 42% (28–78) in the CVF-treated group.

(iv) *Renal-bound radiolabelled BSA in the nephrectomy specimen* (Table 1). Thus, both groups before CVF administration are similar.

Half-disappearance rate of renal-bound BSA (Fig. 1)

The t_{50} values for the CVF-treated group (mean 19·5 days, range 9·3–60) were significantly higher than those observed in the control group (mean 11·3 days, range 8·7–15) ($P < 0{\cdot}01$), using the Wilcoxon rank sum test for two samples. In the CVF-treated groups the lowest t_{50} values occurred in the rabbits (No. 6 and 14) showing the highest plasma concentrations and most extensive glomerular deposits of C3 at autopsy (see Table 3).

Histology (Table 2)

No significant difference was found between the groups in PMN or non-PMN cell count of kidneys taken at nephrectomy or at autopsy. However, the PMN cell count in the nephrectomy specimens in the CVF-treated rabbits were significantly higher ($P < 0{\cdot}01$) than those of the control group.

Immunofluorescence (Table 3, Figs 2 and 3)

No difference in C3 deposition was discernible at nephrectomy, but deposits were substantially less in CVF-treated animals at autopsy. Thus, in the CVF-treated animals trace deposits were found in

TABLE 3. Comparison by immunofluorescence of kidneys obtained at nephrectomy (N) and at autopsy (A)

	IgG		BSA		C3		
Rb	N	A	N	A	N	A	C3 (%)*
Control group							
12	tr.	tr.	0	0	2+	2+G	105
11	1+G	tr.	0	0	2+G	2+G	75
5	tr.	1+	0	0	1+G	2+G	100
2	1+G	tr.G	0	0	3+G	3+G	100
16	tr.	tr.	0	0	1+G	tr.	110
23	1+	tr.	tr.	0	2+	2+G	125
21	1+	1+	tr.	0	1+	2+G	100
22	n.d.	2+	n.d.	0	n.d.	2+G	110
8	1+	0	0	0	3+G	3+G	105
17	tr.	tr.	0	0	2+G	2+	110
CVF group							
10	1+	1+†	0	0	1+	tr.	24
18	1+	1+	0	0	1+G	tr.	14
1	tr.	1+	0	0	2+G	tr.G	21
3	tr.	tr.	0	0	2+G	1+	11
13	tr.	tr.	0	0	2+	tr.	8
19	1+	1+	0	0	2+	tr.	12
25	1+	1+	tr.	0	2+	tr.	3
24	1+	tr.	tr.	0	3+	1+	5
6	1+	tr.	0	0	2+	2+	43
14	2+	1+	0	0	2+	1+	93
20	1+	1+	0	0	2+G	tr.	8

n.d. = Not done; G = globular pattern of deposition; tr. = trace.

* Plasma immunoreactive C3 on day of autopsy, expressed as percentage of C3 at day 4.

† Linear staining of GBM.

TABLE 4. *In vitro* C3 fixation by cryostat sections of autopsy kidneys from decomplemented rabbits (Nos 18, 19 and 25)

Source of complement (20 at 37°C)	Glomerular immunofluorescence					
	Anti-rabbit C3			Anti-guinea-pig C3		
	18	19	25	18	19	25
EDTA–GPS	tr.*	tr.*	tr.*	0	0	0
C4-DGPS	tr.*	tr.*	tr.*	tr.	tr.	tr.†
Mg–EGTA/RS	+	+	+			
RB-RS	+	2+	+			

* Trace amounts of rabbit C3 are already present before incubation (Table 3).

† The scoring is as used earlier, and it is to be emphasized that the amounts of guinea-pig C3 observed in this group were reproducibly and easily distinguishable from the negative staining in the control (EDTA–GPS) experiment.

seven out of eleven rabbits and only one showed a 2+ staining; in control rabbits, nine animals showed 2+ or greater staining and only one showed trace deposits.

The pattern of glomerular deposits of C3 was also different: in control animals eight out of ten specimens obtained at autopsy showed globular deposits of C3, whereas this was only seen in one out of eleven of the CVF-treated group.

No significant difference was detected in the relatively small amounts of IgG deposited in the glomeruli, and BSA could not be detected by immunofluorescence at autopsy in any animal.

Complement fixation by glomerular immune complexes in vitro (Table 4).

The results in Table 4 suggest that C3 of rabbit or guinea-pig origin is fixed to immune complexes lodged *in vivo* in the renal glomerulus by activation of the alternative pathway; it is clear from the results with rabbit RB that classical pathway activation is also produced. C3 was not detected in the glomeruli of normal rabbits when kidney sections were incubated with any of the complement reagents, although a variable amount of C3 was found in the renal tubules of both pathological and normal specimens when incubated with C4-deficient guinea-pig serum.

DISCUSSION

We have shown that CVF-induced hypocomplementaemia has resulted in a significant prolongation of the half-clearance time (t_{50}) of renal-bound, radiolabelled BSA, and that in the course of acute serum sickness, local (glomerular) turnover of deposited complement occurs: CVF was not administered until 4 days after 99% elimination of circulating antigen, so presumably complement is being deposited and removed at this stage, and C3 deposition is prevented by CVF treatment. Our data suggest that the immune complexes deposited in the kidney in the course of acute serum sickness in CVF-treated animals fix C3 via the alternative pathway, since C3 deposition occurred when sections were treated with magnesium– EGTA-treated rabbit serum and C4-deficient guinea-pig serum. Similar observations have been made in human mesangial IgA nephropathy (J. Uff and D. G. Evans, 1977). Thus the tissue-bound complexes appear to have the same capacity to activate complement as the immune precipitates studied *in vitro* (Miller & Nussenzweig, 1975; Czop & Nussenzweig, 1976). These workers showed that this fixation of C3 caused complex desaggregation, probably due to intercalation of C3 molecules into the lattice of the complex, thereby resulting in its rearrangement into smaller and therefore more soluble complexes. These findings thus support our hypothesis that an intact alternative pathway plays a part in the *in vivo* solubilization of glomerular deposits. The depletion of complement that occurs as part of immune complex disease (i.e. complement consumption following an antigen–antibody reaction) might itself lead to abnormal handling of immune complexes in the kidney and elsewhere.

We noted that 3–6 hr after CVF administration, the glomerular PMN count was significantly greater than in control animals. This could have been due to the transient margination of PMNs, which has been described following systemic complement activation (Majewski & Brown, 1976; Fehr & Jacob, 1977), though we find it difficult to connect this observation with the prolongation in t_{50} of deposited BSA in the CVF-treated animals.

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