Immunology 1986 **59** 81–85

Studies in the rat of antibody-coated and *N*-ethylmaleimide-treated erythrocyte clearance by the spleen

II. EFFECTS OF IMMUNE COMPLEX INFUSION

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Accepted for publication 7 May 1986

SUMMARY

The effects of immune complex infusion on the clearance of antibody (R3/13)-coated and NEM-treated rat erythrocytes by the splenic component of the rat mononuclear phagocyte system (MPS) were investigated. Equivalence complexes of BSA–anti-BSA produced a significant delay in the clearance of the NEM cells (pre-infusion $T\frac{1}{2}$ 19·7 ± 3·9 min, post-infusion $T\frac{1}{2}$ 26·5 ± 3·8 min, mean ± SE, $n = 6$, $P < 0.01$), but this effect could be abolished by agents that prevented the changes in the splenic blood flow that followed complement activation. The immune complexes formed in 10-fold antigen excess (mean size 15S) did not delay the clearance of the NEM cells. Clearance of R3/13-coated cells was impaired by the infusion of immune complexes prepared at equivalence, 10-fold antigen excess or complexes prepared with $F(ab')_2$ fragments of rabbit anti-BSA antibody. The inhibition of red cell clearance was independent of changes in blood flow, but the degree of inhibition produced did not correlate well with the dose of immune complex injected.

INTRODUCTION

In the previous paper we have described an experimental model whereby the factors that influence the clearance of antibody-coated and *N*-ethylmaleimide (NEM)-treated erythrocytes by the splenic component of the mononuclear phagocyte system (MPS) could be studied. The major finding to emerge from this study was the demonstration that *in vivo* complement activation led to a significant reduction in splenic blood flow, thereby delaying the clearance of NEM-treated cells from the circulation (Yousaf, Howard & Williams, 1986).

Although there has been one report that shows that passively administered immune complexes formed in antigen excess delay the clearance of NEM-treated cells by the rabbit spleen (Lawrence, Lockwood & Peters, 1981), no attempt was made to eliminate the possibility that the observed effects were mediated by changes in splenic blood flow. This paper examines *in vivo* the effects of immune complex infusion on the immunospecific (C3b) and non-specific erythrocyte clearance by the rat spleen.

MATERIALS AND METHODS

Animals

Male PVG/c and DA rats weighing 160–190 g were obtained from Bantin and Kingman Ltd (Aldbrough, Hull).

Abbreviations: BSA, bovine serum albumin; NEM, *N*-ethylmaleimide.

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Immunoelectrophoresis

Laurell (1966) two-dimensional immunoelectrophoresis was used to demonstrate in rat plasma (collected into EDTA—final molarity 0·02) the activated fragments of C3.

Anti-BSA antibodies

Antisera to crystallized bovine serum albumin (BSA, Sigma Chemical Company Ltd, Poole, Dorset) were raised in rabbits by immunization with 2 mg of BSA emulsified in Freund's complete adjuvant. Repeated booster injections were given at 2-week intervals until precipitating antibody was present in the sera.

Preparation of the $F(ab')_2$ anti-BSA

Protein A (Pharmacia, Uppsala, Sweden)-purified rabbit anti-BSA antibodies were pepsin-digested and $F(ab')_2$ fragments isolated after applying the digest to a G100 Sephadex column. This preparation could still precipitate BSA, and the absence of Fc in the preparation was demonstrated by immunodiffusion against a goat anti-rabbit antiserum that was specific for the Fc portion of rabbit immunoglobulin (Nordic, Maidenhead, Berks).

Radio-iodination

Rabbit anti-BSA antibodies (affinity-purified) or BSA were radiolabelled with ^{125}I using the Iodogen method as described by Fraker & Speck (1978). The reaction was terminated by the addition of sodium iodide to the sample (final molarity 0·25 M). The radiolabelled sample was then extensively dialysed against

PBS, pH 7·2, to remove free iodine and then mixed with unlabelled antigen or antibody. Specific activity ranged from 0·25 to 2 μCi/μg.

Preparation of BSA–anti-BSA immune complexes

Immune complexes were prepared at equivalence and in 10 times antigen excess by incubating the anti-BSA antibodies (acid eluted from protein A) or the $F(ab')_2$ anti-BSA fragments with BSA at 37° for 30 min, then for a further 30 min at 4°. For the antigen excess complexes, any insoluble material was removed by centrifugation at 1500 ***g***. The supernatants were then used in the clearance studies.

Characterization of the complexes by sucrose density ultracentrifugation

In order to determine the characteristics of the complexes, the above procedure was repeated using radiolabelled antigen or radiolabelled antibody and the complexes formed were stored at 4° prior to sucrose density gradient ultracentrifugation. One-hundred μl samples of the immune complex preparation to be injected were applied and linear sucrose density gradient ultracentrifugation performed at 110,000 ***g*** for 18 hr using a prepspin 65 (MSE). Twenty-five fractions, each of 200 μl, were collected and counted in a Gamma Counter and the radioactivity in each fraction plotted as a percentage of the total counts. Iodine-131-labelled normal rabbit IgG and BSA were used as 7S and 4S markers, respectively, and iodine-125-labelled Clq was used as an 11S marker.

Preparation of NEM-treated and R3/13-coated erythrocytes and their clearance in vivo

The *in vivo* clearance of NEM- and R3/13-treated cells was carried out as previously described (Yousaf *et al.*, 1986). Because of the variability in the $T\frac{1}{2}$ of both NEM- and R3/13-coated cells in normal animals, the effects of immune complexes on red cell clearance were determined by infusing the complexes into the tail vein at varying times, usually 15–30 min, after the injection of the radiolabelled cells. The volume injected varied between 0·3 ml and 0·5 ml, and the sample was administered over 5–10 seconds. Controls for this procedure included the injection of equal volumes of saline, BSA and rabbit immunoglobulin. The clearance times ($T\frac{1}{2}$) before and after the infusion were determined by linear regression analysis, and differences between the results obtained were analysed by a paired *t*-test.

When chromium- and technetium-labelled cells were used in the same animal, the appropriate corrections were made for the different half-lives of the two isotopes and the cross-over between the two isotope channels.

RESULTS

Characteristic and tissue distribution of immune complexes

The size distribution of immune complexes prepared at equivalence ranged from 25 to 35S. Antigen-excess immune complexes were smaller, ranging from 10 to 19S, with a predominant size of 15S.

The tissue distribution of immune complexes prepared at equivalence and antigen excess are shown in Table 1. The equivalence complexes were removed rapidly from the circulation, with an initial half-life of less than 5 min. When the animals were killed 35 min after the injection, 49% of the radioactivity was present in the liver and only 3% in the spleen. Antigen-excess complexes were removed much more slowly from the blood in a non-linear fashion, and at 90 min 65% of the radioactivity present 1 min after the injection remained in the circulation. At 170 min the uptake in the liver and spleen was 15% and 0·5%, respectively, the latter value being no greater than that seen when the animals were injected with the radiolabelled antigen. Only 40–67% of the administered radioactivity could be recovered in these studies.

In vivo complement activation produced by the infusion of immune complexes

Activated fragments of C3 were detectable 1–2 min after the infusion of both equivalence and antigen-excess complexes containing 340 μg of specific antibody. Complexes formed at equivalence with 340 μg of the $F(ab')_2$ fragment of the anti-BSA also led to C3 activation, but they were less efficient than the complexes formed with intact antibody. Normal rat plasma, collected into the EDTA, did not show any activated fragments of C3.

The effect of immune complexes on the clearance of NEM-treated erythrocytes

The results of infusing immune complexes, prepared at equivalence, on the clearance of NEM cells are shown in Table 2. A small but significant increase in the $T\frac{1}{2}$ was observed following the infusion, but this was prevented by pretreating the rats with Piriton. Large quantities of antigen-excess immune complexes

Table 1. Tissue distribution of BSA–anti-BSA immune complexes in normal rats

		Percentage of the radioactivity administered				
	n	Lung	Spleen	Kidneys	Liver	Blood
ICs formed at equivalence (500 μg anti-BSA)	5	4·7±0·7	3·6±0·3	2·1±0·7	49·8±5·1	6·8±2·7
ICs prepared at 10 × antigen excess (90 μg anti-BSA)	5	1·5±0·1	0·5±0·1	1·7±0·1	15·5±2·4	32·0±1·9
BSA alone (300 μg)	4	1·0±0·2	0·4±0·04	1·6±0·1	4·4±0·3	21·7±2·3
Anti-BSA alone (250 μg)	3	1·5±0·2	0·5±0·1	1·8±0·2	6·8±0·4	40·9±4·0

Data are given as the mean±SE; *n* represents the number of animals in each group.

Table 2. Effect of BSA–anti-BSA immune complexes on the clearance of NEM-treated cells

		$T\frac{1}{2}$ of NEM-treated cells	
	n	Before infusion	After infusion
ICs prepared at equivalence			
300 μg anti-BSA	6	19·7±3·9	26·5±3·8†
300 μg (rats pretreated with Piriton)	6	18·1±4·2	19·8±4·1
ICs prepared at 10 × antigen excess			
1·2 mg anti-BSA	5	21·1±3·4	17·1±2·6*
Controls			
BSA alone 300 μg	5	24·5±1·6	14·3±1·2†
4 mg	4	24·6±2·2	13·7±0·8†
Anti-BSA alone 340 μg	5	30·0±1·8	18·3±2·0‡

Data are given as the mean ± SE; *n* represents the number of animals in each group.
*$P<0.05$.
†$P<0.01$.
‡$P<0.001$.

Table 3. Effect of BSA–anti-BSA immune complexes on the clearance of R3/13 antibody-sensitized cells

		$T\frac{1}{2}$ of R3/13 antibody-coated cells	
	n	Before infusion	After infusion
ICs prepared at equivalence			
40 μg anti-BSA	5	54·8±8·4	67·5±7·1
90 μg	5	57·4±5·2	75·8±6·7*
270 μg	7	27·8±3·5	46·7±2·4†
220 μg (rats pretreated with Piriton)	5	39·1±7·7	65·9±7·9‡
ICs prepared at 10 × antigen excess			
90 μg anti-BSA	5	37·6±2·7	65·0±10·0*
170 μg	5	32·1±8·8	58·6±4·3§
1200 μg	5	38·9±5·2	64·3±6·0§
Controls			
BSA alone 300 μg	5	53·1±6·0	51·5±5·8
4 mg	5	47·1±3·0	48·0±3·1
Anti-BSA alone 330 μg	5	54·5±4·7	56·7±3·7
1·2 mg	4	46·4±3·9	53·0±4·5

Data are given as the mean ± SE; *n* represents the number of animals in each group.
*$P<0.05$.
†$P<0.02$.
‡$P<0.01$.
§$P<0.001$.

did not increase the $T\frac{1}{2}$ of these cells. In the control rats receiving either BSA or anti-BSA, the clearance of these cells was significantly faster after the infusion of both of these proteins.

The effects of immune complexes on the clearance of R3/13 cells

The results of administering immune complexes on the clearance of R3/13 cells in normal rats are shown in Table 3. With the complexes formed at equivalence, infusion of as little as 90 μg of complexed antibody was capable of producing a significant increase in the $T\frac{1}{2}$. Increasing the concentration of the antibody to 270 μg produced a larger increment in the $T\frac{1}{2}$, but the significance of this was doubtful because of the difference in the initial $T\frac{1}{2}$ of the two groups.

Antigen-excess complexes given over a dose of 90–1200 μg of anti-BSA antibody also produced a significant increase in the $T\frac{1}{2}$ of the R3/13-coated cells. In the animals receiving either comparable amounts of antigen or antibody alone, the $T\frac{1}{2}$ before and after the infusion did not change significantly.

The non-specific effects of blood flow changes triggered by complement activation could best be demonstrated by observing the effect of immune-complex infusion on the clearance of NEM-treated and R3/13 antibody-sensitized cells injected together into the animal. Figure 1 demonstrates that the clearance of both cells is delayed following the infusion of equivalence complexes. Pretreatment of the rat with Piriton prior to the administration of immune complexes abolishes their effect on the clearance of NEM-treated cells, but leaves unaltered the increase in the $T\frac{1}{2}$ of the R3/13 cells (Fig. 2).

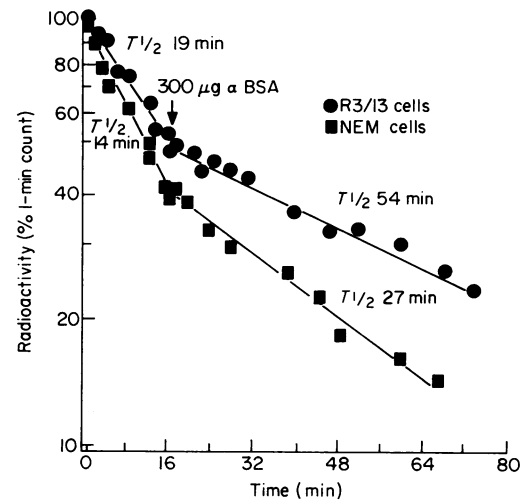

Figure 1. Effect of immune complexes (formed at equivalence containing 300 μg of anti-BSA) on the clearance of R3/13 antibody-sensitized and NEM-treated erythrocytes in a normal rat. Arrow indicates the point at which the complexes were injected.

Effects of BSA F(ab′)$_2$ anti-BSA complexes

Because of their ability to initiate complement activation, both antigen-excess and equivalence complexes formed with the F(ab′)$_2$ fragments were able to prolong the clearance of the R3/13 cells in normal rats (Fig. 3). The quantities required to produce this effect, for both immune complexes, were much greater than those observed with the complexes formed with intact antibody. The infusion of complexes formed with the F(ab′)$_2$ fragment of rabbit antibody has no effect on the clearance of NEM-treated cells (data not shown).

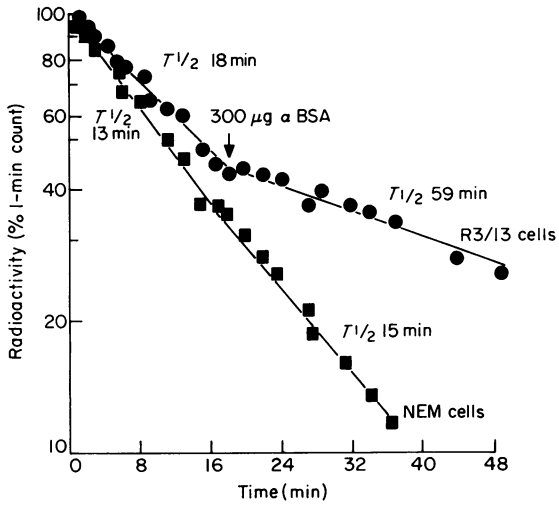

Figure 2. Effect of immune complexes (formed at equivalence containing 300 μg of anti-BSA) on the clearance of R3/13 antibody-sensitized and NEM-treated erythrocytes in a rat pretreated with 0·5 mg Piriton.

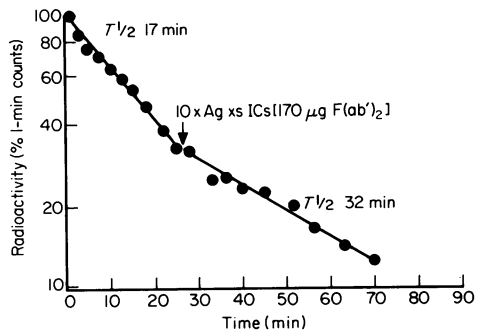

Figure 3. Effect of immune complexes (formed at 10-fold antigen excess containing 170 μg of $F(ab')_2$ fragment of anti-BSA) on the clearance of R3/13 antibody-coated erythrocytes in a normal rat.

DISCUSSION

This work examines directly the effects of immune complex infusion on the clearance of antibody-coated and chemically modified cells by the rat spleen. Earlier experiments have established that the clearance of both cell suspensions is highly spleen dependent, and that the removal of the R3/13 cells occurs through the interaction of cell-bound C3b and complement receptors on the splenic macrophages.

Although our studies provide direct evidence to suggest that the clearance of these two particles can be influenced by passively administered immune complexes, they indicate that the mechanisms that lead to inhibition are different. Once changes in the splenic blood flow are prevented from taking place, any effect of immune complex infusion in normal rats on the clearance of NEM-treated cells was lost. In contrast to other published work (Lawrence *et al.*, 1981), we were unable to show any effect of antigen-excess immune complexes on the clearance of NEM-treated cells, even though, on a body weight basis, the dose used was sufficient to delay the clearance of NEM-treated erythrocytes by the rabbit spleen. Since histamine release from rabbit platelets is known to occur following their interaction with immune complexes (Cochrane & Koffler, 1973), it is possible that the differences in our findings reflect differences in the sensitivity of the splenic microcirculation in these two animals to the effects of histamine release.

The clearance of antibody-coated cells by complement receptors was susceptible to inhibition by both types of immune complexes. The inhibition in red cell clearance occurred quickly after complex infusion, but had little relationship to the quantity of immune complex in the dose range injected. With the antigen-excess complexes, a 13-fold increase in the quantity injected produced no apparent increment in the degree of inhibition of erythrocyte clearance. Our studies using complexes formed with the $F(ab')_2$ fragments of rabbit antibody, which activate the alternative pathway (Reid, 1971), suggest that inhibition of erythrocyte clearance can be achieved either by the generation of fluid phase fragments of C3, or by C3 bound to $F(ab')_2$ complexes. These fragments are known to bind to the C3b receptor (Berger *et al.*, 1981), and we presume that they impair red cell clearance by competing with erythrocyte-bound C3b or iC3b for the macrophage complement receptor(s). It is likely, since immune complexes containing C3 also bind to complement receptors (Eden, Bianco & Nussenzweig, 1973, Theofilopoulos *et al.*, 1974), that the complexes in the circulation and those bound to the splenic macrophage are also capable of interfering with the attachment of erythrocytes to the macrophage surface.

It has been proposed that studies on non-specific and receptor-specific erythrocyte clearance by the spleen are of value in immunological disease in man, since they identify defects in the reticuloendothelial system that may be associated with a continued circulation of immune complexes. We have been unable to obtain any direct evidence to support the view that the interaction of immune complexes with splenic macrophages can be reflected by changes in the clearance of chemically modified cells. As a result of these experiments with NEM-treated erythrocytes, we think it likely that the delayed clearance of heat-damaged erythrocytes observed in man (Williams *et al.*, 1979) arises secondarily to changes in splenic blood flow. It has not been possible to test this directly in this system because of the difficulties in producing uniform and reproducible heat damage to rat red cells. Complement-mediated clearance of sensitized erythrocytes by the rat spleen can be influenced by the infusion of immune complexes. However, the increase in the $T\frac{1}{2}$ of these cells is seldom more than three times the initial clearance rate and correlates poorly with the quantities of immune complexes present in serum. This poor correlation is consistent with the idea that maximum inhibition of splenic clearance occurs at relatively low concentrations of immune complex, and that once saturation occurs, subsequent immune clearance of the erythrocytes occurs via a complement-independent receptor.

ACKNOWLEDGMENTS

This work was supported by a grant from the Arthritis and Rheumatism Council, and in part by USPHS Project Grant CA 34913 to J. C. Howard.

REFERENCES

BERGER M.B., GAITHER T.A., MANNER C.H. & FRANK M.K. (1981) Lack of binding of human C3 in its native state, to C3b receptors. *J. Immunol.* **127**, 1329.

COCHRANE C.G. & KOFFLER D. (1973) Immune complex diseases in experimental animals and man. *Adv. Immunol.* **16,** 185.

EDEN A., BIANCO C. & NUSSENZWEIG V. (1973) C3 split products inhibit the binding of antigen–antibody–complement complexes to B lymphocytes. *J. Immunol.* **110,** 1452.

FRAKER P.J. & SPECK J.C. (1978) Protein and cell membrane iodinations with a sparingly soluble chloramide, 1,3,4,6,-tetra-chloro-3a, 6a diphenylglycoluril. *Biochem. biophys. Res. Commun.* **80,** 849.

LAWRENCE S., LOCKWOOD C.M. & PETER D.K. (1981) Studies on NEM treated erythrocyte clearance in the rabbit, with special reference to the effects of circulating immune complexes. *Clin. exp. Immunol.* **44,** 433.

LAURELL C.B. (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Analyt. Biochem.* **15,** 45.

REID K.B.M. (1971) Complement fixation by the $F(ab')_2$ fragment of pepsin treated rabbit antibody. *Immunology,* **20,** 649.

THEOFILOPOULOUS A.N., WILSON C.B., BOKISH V.A. & DIXON F.J. (1974) Binding of soluble immune complexes to human lymphoblastoid cells. II. Use of Raji cells to detect circulation immune complexes in animal and human sera. *J. exp. Med.* **140,** 1230.

WILLIAMS B.D., PUSSELL B.A., LOCKWOOD C.M. & COTTON C. (1979) Defective reticuloendothelial system function in rheumatoid arthritis. *Lancet,* **i,** 1311.

YOUSAF N., HOWARD J.C. & WILLIAMS B.D. (1986) Studies in the rat of antibody coated and *N* ethylmaleimide treated erythrocyte clearance by the spleen. I. The effects of *in vivo* complement activation. *Immunology,* **59,** 75.