Clin. exp. Immunol. (1986) **66**, 654–660

Studies in cobra venom factor treated rats of antibody coated erythrocyte clearance by the spleen: differential influence of red blood cell antigen number on the inhibitory effects of immune complexes on Fc dependent clearance

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SUMMARY

The splenic component of the mononuclear phagocyte system (MPS) was investigated in decomplemented rats by determining the clearance from the blood of erythrocytes coated with a monoclonal antibody (R3/13). The infusion of immune complexes (IC), prepared at 10-fold antigen excess, at an appropriate time during the erythrocyte clearance produced a significant increase in the $T\frac{1}{2}$ of the antibody coated cells. Immune complexes formed with the $F(ab')_2$ fragment of the rabbit antibody did not have any significant effect. A positive correlation was seen between the dose of immune complex infused and the degree of inhibition of erythrocyte clearance. The influence of red cell antigen number on the behaviour of erythrocytes sensitized with R3/13 was studied by comparing the clearance of DA and (DA × PVG) F1 erythrocytes. F1 erythrocytes, with only half the number of specific antigens on their surface that bind R3/13 antibody were cleared much more slowly ($82 \pm 2{\cdot}6$ min, mean ± s.e.) by the spleen than the DA erythrocytes ($44 \pm 1{\cdot}5$ min $P < 0{\cdot}001$). Both cell suspensions were equally susceptible to inhibition by soluble IC. These studies show that the number of specific antigens on the red cell surface influences the rate at which sensitized cells are removed by splenic macrophage Fc receptors but not their susceptibility to inhibition by IC. Our results draw attention to a major defect in the use of autologous erythrocytes coated with anti-rhesus (D) immunoglobulin to assess macrophage Fc receptor function in man.

Keywords mononuclear phagocyte system Fc receptor immune complex

INTRODUCTION

It is an attractive hypothesis that in humans with immune complex disease the reticuloendothelial system (RES) has become saturated and is therefore unable to clear immune complexes efficiently from the circulation. Indirect evidence which supports this idea has been derived from studies in man which have measured the clearance from the blood of autologous erythrocytes coated with an IgG anti rhesus (D) antibody. Impaired clearance of these cells has been interpreted as reflecting defective splenic macrophage Fc function in a number of immune complex mediated diseases (Frank *et al.*, 1979; 1983; Lockwood *et al.*, 1979; Hamburger *et al.*, 1979; Parris *et al.*, 1982).

All of these studies have ignored the possibility that some property of the infused red cell may also influence their clearance *in vivo*. Studies which have compared Fc function between patients

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and control subjects have assumed that the probe used is homogenous and that differences in the clearance rates reflect differences in macrophage Fc function. However, it has recently been shown that differences in the distribution of the rhesus (D) antigen on the red cell surface can markedly influence the clearance rate of these cells *in vivo* (Williams, O'Sullivan & Ratanachaiyavong, 1985).

There is no direct evidence available which shows that the splenic clearance of IgG coated cells can be influenced by the presence of circulating immune complexes. This is an important omission since most of the experimental studies which have examined RES function in animals have demonstrated that preformed immune complexes or IgG aggregates are removed from the circulation by the liver, with the spleen playing only a minor role (Haakenstad & Mannik, 1974; Finbloom & Plotz, 1979; Bockow & Mannik, 1981). In this study, using methods which assess splenic macrophage function in the rat (Yousaf, Howard & Williams, 1986a, b), we have examined directly whether Fc specific clearance could be inhibited by passively administered immune complexes. Since erythrocytes from (PVG × DA) F_1 rats have half the number of antigens on the cell surface which bind the sensitizing antibody (Howard *et al.*, 1979) we were able to compare the clearance rates of these cells with the DA cells and investigate any differential effect of immune complex infusion on their clearance by the spleen.

MATERIALS AND METHODS

Male PVG/c and DA rats weighing 170–200 g were obtained from Bantin and Kingman Ltd (Aldbrough, Hull) and (DA × PVG)F_1 rats from ARC, Institute of Animal Physiology, Babraham.

Isolation of F(ab')$_2$ anti BSA. To prepare F(ab')$_2$ fragments rabbit anti BSA antibodies (acid eluted from protein A) were digested with pepsin and fractionated by chromatography on Sephadex G-100. This preparation could still precipitate BSA and was shown to contain no detectable Fc activity when analysed by immuno-diffusion against a goat anti rabbit anti serum which was specific for the Fc portion of the rabbit IgG (Nordic).

Preparation of immune complexes. BSA anti-BSA immune complexes (IC) were prepared at ten times antigen excess as previously described (Yousaf, Howard & Williams, 1986b). Briefly, protein A purified anti-BSA antibodies or their F(ab')$_2$ fragments were incubated with an appropriate amount of BSA at 37°C for 30 min and then for a further 30 min at 4°C. Before their use in the clearance studies any insoluble material was removed by centrifugation at 4,000 rev/min. The size distribution of the IC formed with the intact anti-BSA antibody ranged from 10S to 19S with the predominant size being 15S.

Heat aggregated human gamma globulin. HAGG was prepared by the method of Greenwood *et al.* (1971). The size of the aggregates prepared in this manner ranged from 30S to 35S and the aggregated Ig G remained soluble and stable when stored at −70°C.

Radio-iodination. Affinity purified anti-BSA antibodies and HAGG were radiolabelled with ^{125}I using the iodogen technique as described by Fraker & Speck (1978).

Complement depletion of animals with cobra venom factor. Cobra venom factor (CVF) was prepared from Naja Naja venom (Sigma) by the method of Lachmann & Hobart (1978). Rats were decomplemented by the intravenous injection of 40 units of CVF 20–24 h before the clearance studies. The C3 levels at the time of the study were less than 5% of that seen in normal rat plasma.

Sensitization of erythrocytes. Erythrocytes obtained from DA and (DA × PVG)F_1 rats were radiolabelled with ^{51}Cr and ^{99m}Tc respectively and then sensitized with the same amount of R3/13 antibody in the manner described previously (Yousaf, Howard & Williams, 1986a).

Clearance of R3/13 antibody coated cells. The clearance studies were performed in PVG/c rats. The procedure adopted for measuring the clearance *in vivo* of these cells has been reported earlier (Yousaf, Howard & Williams, 1986a). For the studies which examined the inhibitory effects of immune complexes or HAGG, these substances were administered intravenously at an appropriate time after the injection of erythrocytes. The clearance times ($T^{\frac{1}{2}}$) before and after the infusion of the immune complexes were calculated separately for each animal and the significance of the differences observed between the pre and post infusion values were calculated using a paired *t*-test.

RESULTS

Blood clearance and tissue distribution of immune complexes and HAGG. Antigen excess complexes (mean size 15S) were removed slowly and in a non-linear fashion from the blood of decomplemented animals, 63·8% ± 2·2% (mean ± s.e., $n = 9$) of the radioactivity remaining in the circulation at 90 min. When the animals were killed at 150 min the blood level had fallen to 56·0 ± 2·7%. Most of the injected complexes had accumulated in the liver although there was a significant uptake by the spleen (Table 1).

The removal of HAGG from the circulation was dependent on the injected dose (data not shown). The clearance was again non-linear and even when 4·5 mg was injected 50% of the HAGG had disappeared from the blood within 9 min. When animals were killed 50 min after the injection of HAGG, 34% of the injected radioactivity was present in the liver and only 0·6% in the spleen (Table 1). At 90 min the level of radioactivity had changed, the liver had fallen to 9·9%, splenic uptake was 0·7% but the amount present in the kidneys had increased 5-fold to 7·3%.

Splenic clearance of R3/13 antibody coated DA erythrocytes in CVF treated rats. Analysis of the tissue distribution of R3/13 antibody coated erythrocytes in CVF treated animals established that the clearance of these cells was dependent on the spleen (Yousaf, Howard & Williams, 1986a). Their clearance was significantly delayed in decomplemented splenectomized rats ($T^{\frac{1}{2}} > 180$ min).

Clearance times $T^{\frac{1}{2}}$ of DA and (DA × PVG) F_1 erythrocytes sensitized with R3/13. In the group of 36 animals the $T^{\frac{1}{2}}$ of the DA erythrocytes, 44 min ± 1·5 minutes (mean ± s.e.) was significantly faster than that of the (DA × PVG)F_1 erythrocytes, 82·0 min + 2·6 min (mean ± s.e.) $P < 0·001$ (Fig. 3).

Changes in the clearance of R3/13 coated DA cells following the infusion of immune complexes. Figure 1 demonstrates clearly that the inhibition of R3/13 antibody sensitized-erythrocyte clearance in decomplemented animals is Fc specific since F(ab′)$_2$ antigen excess immune complexes had no effect on the clearance of these cells. In normal rats F(ab′)$_2$ complexes are capable of delaying the removal of R3/13 sensitized cells by initiating complement activation and generating fragments of C3 (Yousaf, Howard & Williams, 1986b). Inhibition of clearance could be produced by very small quantities of antigen excess IC (containing 90 μg of specific antibody) and a positive correlation existed between the dose infused and the degree of inhibition of red cell clearance.

The effect of immune complexes and HAGG on the clearance of R3/13 coated DA and (DA × PVG) F_1 erythrocytes. Figure 2 shows the effect of infusing 400 μg of antigen excess immune complexes on the clearance of R3/13 antibody sensitized DA and (DA × PVG) F_1 erythrocytes in a

Table 1. Tissue distribution of BSA anti-BSA immune complexes and HAGG in CVF-treated rats

		Per cent of the radioactivity administered				
	n	Lung	Spleen	Kidneys	Liver	Blood
BSA anti-BSA IC						
90 μg anti-BSA (rats killed at 150 min)	4	1·5 ± 0·1	1·0 ± 0·1	2·4 ± 0·3	14·4 ± 1·5	40·9 ± 3·3
BSA						
300 μg (rats killed at 150 min)	5	1·0 ± 0·04	0·4 ± 0·03	1·6 ± 0·03	4·3 ± 0·2	19·5 ± 0·5
HAGG						
4·5 mg (rats killed at 50 min)	4	1·2 ± 0·1	0·6 ± 0·1	1·5 ± 0·2	34·2 ± 3·3	12·4 ± 1·4
4·5 mg (rats killed at 90 min)	4	0·8 ± 0·1	0·7 ± 0·05	7·3 ± 0·8	9·9 ± 0·2	3·2 ± 0·1

Mean ± s.e.
n represents the number of animals in each group.

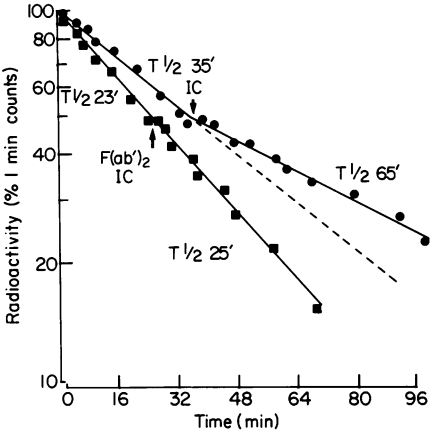

Fig. 1. Effect of antigen excess immune complexes (prepared with intact and $F(ab')_2$ rabbit anti BSA) on the clearance of R3/13 antibody sensitized erythrocytes in cobra venom factor treated rats. Animals received 150 μg of specific anti-BSA antibody.

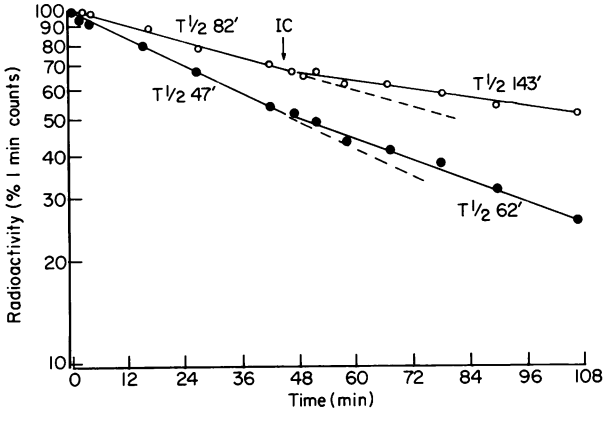

Fig. 2. Effects of immune complexes prepared at × 10 antigen excess containing 400 μg of the anti BSA on the clearance of DA (●) and (DA × PVG) F_1 (○) erythrocytes sensitized with R3/13 antibody in a cobra venom factor treated rat.

single rat. Non-specific events related to the infusion or differences in the activity of the RES of different animals are therefore eliminated and a direct comparison of the effects of immune complexes on Fc mediated clearance can be made. The clearance rates are slower for (DA × PVG) F_1 cells and the difference between the pre and post immune complex infusion $T^{\frac{1}{2}}$ are substantially greater with these cells than the DA cells.

In Figure 3 and 4 we compare the effects of three doses of antigen excess immune complexes and two doses of HAGG on Fc dependent clearance. With the antigen excess IC a clear relationship was observed using both cell suspensions between the dose of immune complexes administered and the increase seen in the post-infusion $T^{\frac{1}{2}}$.

This effect however was always more marked with the (DA × PVG) F_1 erythrocytes. Although the infusion of 1·6 mg of HAGG did not lead to a statistically significant increase in the post-infusion $T^{\frac{1}{2}}$ of the DA erythrocytes it did produce a significant increase in the post infusion $T^{\frac{1}{2}}$ of the F_1 erythrocytes. The infusion of 4 mg of BSA or 1 mg anti-BSA, doses which correspond to the

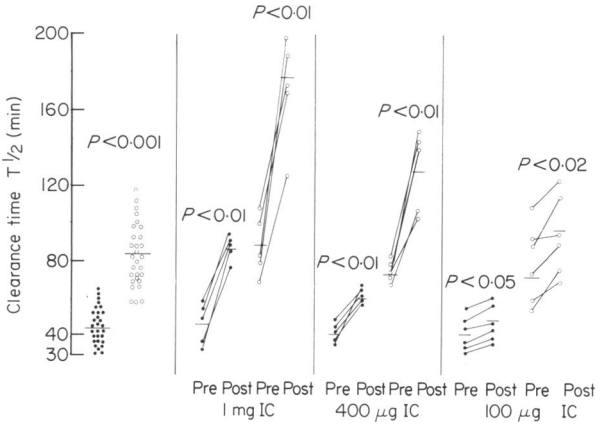

Fig. 3. Comparison of the clearance times ($T^{\frac{1}{2}}$) of DA (●) and (DA × PVG) F_1 (○) erythrocytes sensitized with R3/13 antibody in cobra venom factor treated rats, and the effects of immune complex infusion on the clearance rate. Animals received × 10 antigen excess BSA anti BSA IC at an appropriate time during the erythrocyte clearance.

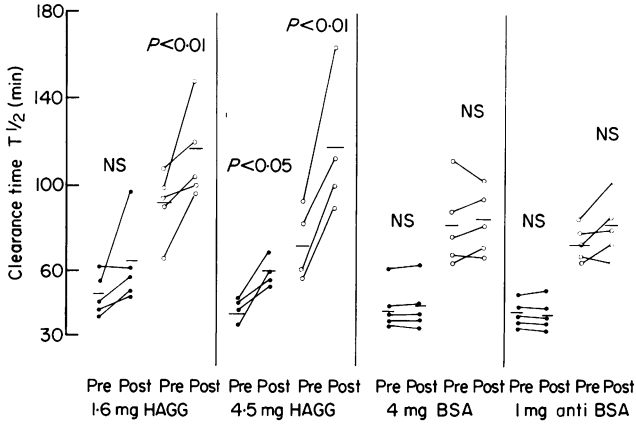

Fig. 4. Effects of HAGG , BSA and rabbit anti BSA on the clearance of DA (●) and (DA × PVG) F_1 (○) erythrocytes sensitized with R3/13 antibody in cobra venom factor treated rats.

maximum quantities used in this study, had no significant effect on the post-infusion clearance times.

DISCUSSION

The infusion of preformed soluble immune complexes into decomplemented rats provides a method of examining directly whether IC can modify the splenic clearance of erythrocytes sensitized with antibody. Since the techniques we developed to assess splenic macrophage function in the rat are very similar to those used in man the information derived from this study would be of value in interpreting the significance of the clearance abnormalities observed in patients with a variety of immune complex-mediated diseases. We did not consider the use of heterologous antibody to prepare the immune complexes to be inappropriate since a high degree of binding occurs between

rabbit immunoglobulin and the rat macrophage Fc receptor (Boltz-Nitulescu, Bazin & Spiegelberg, 1981).

Our results show that small, antigen excess IC are capable of delaying the clearance of antibody-coated erythrocytes by the spleen and that the inhibition of erythrocyte clearance is dependent on the Fc portion of antibody. Studies which have investigated the attachment and ingestion of sensitized erythrocytes by macrophages have shown that their attachment is initially dependent on multiple areas of close membrane contact between the macrophage membrane and the red cell surface (Munthe-Kaas, 1976; Griffin *et al.*, 1975; Kaplan, 1977). Immune complexes could interfere with erythrocyte clearance by competing directly with the red cell bound immunoglobulin for the macrophage Fc receptor. The observation that inhibition of erythrocyte clearance occurred quickly following the infusion of IC and its relationship to the dose infused would support this view.

The way in which complexes of BSA and anti-BSA are processed after binding to the macrophage membrane may explain why very small quantities of these complexes lead to effective inhibition of erythrocyte clearance. It is known that when the complexes of BSA and anti-BSA bind to the macrophage membrane a rearrangement occurs in their distribution (Leslie, 1982). Localized aggregates form at the cell surface because of further cross-linking between IC bound antibody and the antigen in membrane bound complexes. This cross linking and redistribution of IC on the macrophage surface occurs quickly, favours the occupancy of Fc receptors by cell bound IC and leads eventually to a redistribution in macrophage Fc receptors. Since multiple Fc attachments are required for red cell binding, this phenomenon would make this process much less effective. The failure of the IgG aggregates to initiate cross linking once they have become attached to the membrane Fc receptors, coupled with the rapid rate at which they are ingested and degraded after binding, could account for their much less efficient inhibition of erythrocyte clearance.

A comparison of the behaviour *in vivo* of DA and (DA × PVG)F_1 erythrocytes sensitized with antibody and injected into the same animal provides a good method for investigating the influence of antigen density on the rate of removal of erythrocytes by the rat spleen. Halving the number of antigens on the erythrocyte surface significantly reduces the clearance rate of these cells and in every experiment where their clearance was compared with DA erythrocytes the clearance time of the F_1 cells was much slower. The apparently more dramatic effects of IC infusion on the clearance of the F_1 erythrocytes was only due to their initial slower rate of removal and not to any alteration in susceptibility to inhibition by soluble IC.

The range of clearance times ($T^{\frac{1}{2}}$) seen with sensitized DA and F_1 erythrocytes (32–118 min) is comparable to that seen in normal human volunteers injected with autologous erythrocytes sensitized with an anti-rhesus (D) IgG antibody (24–150 min) (Williams, O'Sullivan & Ratanachaiyavong, 1985). Since considerable variability also exists in the number of (D) antigens on the erythrocytes of rhesus positive individuals it is apparent that clearance values outside the normal range, and by inference defective Fc function, will be most easily shown using cells with the lowest surface antigen number. It is also likely that in patients whose red cells have the largest number of D antigens it will not be possible to show significant prolongation of erythrocyte clearance even though the appropriate type of IC are present in the circulation.

It is known in man that different red cell suspensions, even though sensitized with antibody in an identical manner, are removed *in vivo* at very different rates (Williams, O'Sullivan & Ratanachaiyavong, 1985). It has also been observed that when a single source of red cells is used to assess Fc function in patients with immune complex disease putative macrophage Fc receptor defects are no longer demonstrable (O'Sullivan, Walker & Williams, 1985; van der Woude *et al.*, 1984). These observations, taken in conjunction with the experiments reported in this paper, challenge the view that delayed clearance of antibody coated erythrocytes reflect abnormalities in macrophage Fc receptor function. If our experimental findings are relevant they do indicate however that delayed Fc dependent erythrocyte clearance can be produced *in vivo* by small circulating antigen excess immune complexes.

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).

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