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

I. EFFECTS OF *IN VIVO* COMPLEMENT ACTIVATION

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SUMMARY

The splenic component of the mononuclear phagocyte system (MPS) was investigated in the rat using *N*-ethylmaleimide-treated erythrocytes (NEM) and erythrocytes coated with a monoclonal IgG2b antibody (R3/13) directed against the rat RT1A^a major histocompatibility antigen. Both cell suspensions were removed by the spleen, and their clearance times were significantly longer in splenectomized animals. The mean clearance times for the NEM-treated cells in both normal and cobra venom-treated rats were similar ($19{\cdot}1 \pm 1{\cdot}1$ min and $19{\cdot}0 \pm 1{\cdot}0$ min, respectively) but differences were seen between the clearance of R3/13 antibody-sensitized cells in these two groups (normal rats $38{\cdot}3 \pm 2{\cdot}8$ min and CVF-treated rats $51{\cdot}7 \pm 4{\cdot}2$ min, $P < 0{\cdot}02$). Different receptors were also involved in the removal of these cells; in normal animals recognition entailed interaction with complement receptors, whereas in CVF-treated animals this was implemented by Fc receptors. Complement activation prolonged the clearance rates of both R3/13 cells and NEM cells in normal animals, but the effect of complement activation on the clearance of NEM-treated cells was achieved via changes in splenic blood flow. When this was prevented from taking place no effect was seen on the clearance of NEM cells, although the clearance of R3/13 cells was inhibited by the complement fragments generated by complement activation.

INTRODUCTION

The role played by specific membrane receptors (C3b and Fc) in the attachment and ingestion of immune complexes by monocytes and macrophages has been demonstrated by several *in vitro* studies (Mantovani, Rabinovitch & Nussenweig, 1972; Ehlenberger & Nussenzweig, 1977; Arend & Massoni, 1981). Their importance in clearing immune complexes *in vivo* is less well established, although recently there has been considerable interest in the role that defective receptor function might have in aggravating the tissue injury that is associated with a variety of immune complex diseases.

A number of probes have been developed to examine receptor-specific and non-specific reticuloendothelial system (RES) function in humans. Heat-damaged (Pettit, 1977; Williams *et al.*, 1979) and IgG-coated autologous erythrocytes (Frank *et al.*, 1979; Lockwood *et al.*, 1979) are rapidly removed from the circulation and accumulate within the spleen. A number of autoimmune diseases, particularly those associated with the deposition of immune complexes in major organs, are associated with clearance defects. Correlations have been established between the degree of splenic hypofunction and the level of immune complexes, and also between the magnitude of the clearance defect and the severity of the disease process (Frank *et al.*, 1983).

As a result of these studies in man, it is now believed that defects in the function of receptors involved in the recognition and removal of immune complexes occur commonly in patients, and that they contribute to a failure of immune complex removal and enhanced complex deposition in tissues. Despite these conclusions, a number of controversial issues remain unresolved. It is not known if the phenomenon under study is truly a macrophage-associated receptor defect, or if the mechanism that leads to the delayed clearance of antibody-coated cells is similar to that responsible for the prolonged clearance of heat-damaged cells.

In this and succeeding papers, we have attempted to answer these questions by examining in the rat the factors that influence the clearance of antibody-coated and chemically modified cells by the spleen. The model system that we have developed closely

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Abbreviations: CVF, cobra venom factor; i.v., intravenously; NEM, *N*-ethylmaleimide.

parallels the methodology that has been used in man to assess splenic RES function. However, since it proved impossible to damage rat cells in a reproducible manner by heating, the rat erythrocytes were treated with the appropriate concentrations of the sulphydry inhibitor *N*-ethylmaleimide to produce cells that were selectively removed by the splenic microcirculation (Jacob & Jandl, 1962).

MATERIALS AND METHODS

Animals

Male PVG/c and DA rats weighing 160–190 g were obtained from Bantin and Kingman Ltd (Aldbrough, Hull). Splenectomized or sham-splenectomized animals were used 4 weeks after surgery.

Radionuclides

Sodium pertechnetate (Na $^{99m}TcO_4$) in mormal saline was obtained from the Medical Physics Department (Hammersmith Hospital and UHW). Sodium chromate (Na_2 $^{51}CrO_4$) and rubidium chloride (^{86}RbCl) were obtained from the Radiochemical Centre, Amersham, Bucks.

Collection and labelling of erythrocytes with ^{99m}Tc and ^{51}Cr

Rat blood was obtained by cardiac puncture and collected into ACD (acid citrate dextrose). After centrifugation at 400 ***g*** for 8 min the plasma and buffy coat were discarded and the erythrocytes washed three times in 0·85% saline. One-hundred μl of packed cells were labelled with 32 μCi of ^{99}Tc in 320 μl saline at room temperature for 15 min. One-hundred μl of freshly prepared stannous chloride (1 μl/ml) were then added to the cells and the mixture incubated for a further 10 min. Cells were washed twice in saline and resuspended in saline as a 10% suspension (v/v). One-hundred μl of packed cells were incubated with 32 μCi of ^{51}Cr in a volume of 100 μl for 30 min at room temperature. The cells were washed twice and then resuspended in saline at a concentration of 10%.

Sensitization of rat erythrocytes with R3/13 antibody

The rat monoclonal antibody R3/13 (IgG2b) recognizes the P site of RT1A^a class 1 major histocompatibility antigen and activates complement *in vitro* (Howard *et al.*, 1979). Five-hundred μl of a radiolabelled suspension of DA erythrocytes (10%) were incubated with 200 μl of R3/13 antibody at 4° for 60 min. The cells were then washed twice and resuspended at their original concentration in saline.

Treatment of cells with N-ethylmaleimide (NEM)

Two-hundred μl of a 50% (volume/volume) PVG/c erythrocyte suspension in saline was mixed with an equal volume of freshly prepared NEM (3 mg/ml). The mixture was incubated at 37° for 60 min with occasional mixing, washed twice in saline and then labelled with either ^{51}Cr or ^{99m}Tc. After the labelling procedure they were washed three times in saline.

Purification of cobra venom factor

Cobra venom factor (CVF) was purified from Naja Naja venom (Sigma, Poole, Dorset) by DEAE cellulose ion exchange chromatography followed by G200 Sephadex gel filtration (Lachmann & Hobart, 1978). CVF was heat-inactivated by incubation at 70° for 30 min (Lachmann *et al.*, 1976). The rats were decomplemented by the intravenous injection of 40 units of CVF 24 hr prior to the experiment. The C3 levels were less than 5% of those seen in normal animals.

Clearance of R3/13-sensitized and NEM-treated erythrocytes

PVG/c rats were anaesthetized by using anaesthetic ether. One-hundred μl of a 10% cell suspension were injected into the tail vein and 20-μl blood samples obtained at various time-intervals from the tail using a 0·02 ml pipette (Volac). The samples were quickly dispensed into scintillation vials containing 1 ml of saline, 0·02 M EDTA, pH 7·2. When neccessary, the animals were anaesthetized, killed by exsanguination and the organs removed, washed free of blood, dried on filter paper and weighed. The radioactivity in the tissues and blood samples was determined by counting in an LKB CompuGamma and, where appropriate, correction was made for the crossover between the chromium and technetium channels and for the decay of the isotopes.

Determination of organ blood flow

Animals were injected (i.v.) with a known quantity of ^{86}RbCl and killed 60 seconds later by the intravenous injection of 0·5 ml of saturated KCl (Sapirstein, 1958). The radioactivity within the various tissues was determined and the results expressed as the percentage of the cardiac output per gramme of tissue.

Analysis of data

The radioactivity present in the first blood sample, obtained 1 min after the injection of erythrocytes, was taken as the 100% value. The results of the clearance studies were expressed as $T\frac{1}{2}$, the time in minutes required for 50% of the radiolabelled cells to leave the circulation. The $T\frac{1}{2}$ was calculated by linear regression analysis. The significance of differences observed between the various experimental and control groups was analysed using a Student's *t*-test. During the inhibition studies, the $T\frac{1}{2}$ was calculated separately before and after the administration of the relevant agent. The statistical significance of differences observed between the pre- and post-infusion values was again determined by a paired *t*-test.

RESULTS

Survival of normal erythrocytes

Untreated PVG/c or DA erythrocytes, labelled with either ^{99m}Tc or ^{51}Cr, remained in the circulation. At 150 min, more than 90% of the counts present in the sample obtained 1 min after the infusion of the cells remained in the circulation. The organ distribution of these cells is shown in Tables 1 and 2.

The clearance and organ distribution of NEM-treated erythrocytes

PVG/c erythrocytes treated with NEM were cleared from the circulation of normal rats in an exponential fashion with a half-life ($T\frac{1}{2}$) of 19·1 ± 1·1 min (mean ± SE, $n = 28$, range 14–31 min). Their clearance in cobra venom factor-treated animals was similar, the $T\frac{1}{2}$ being 19·0 ± 1·0 (mean ± SE, $n = 22$). The survival of these cells was significantly increased in splenectomized animals, with the $T\frac{1}{2}$ increasing to 39·5 ± 2·0 min (mean ± SE, $n = 13$, $P < 0·001$). Specific accumulation of radioactivity was only seen in the spleen and the liver (Table 1).

Table 1. Tissue distribution of untreated and NEM-treated erythrocytes

	Percentage of the radioactivity administered				
	Lung	Spleen	Kidneys	Liver	Blood
Untreated RBC ($n=5$)	1·3±0·2	0·9±0·04	0·9±0·1	3·2±0·4	35·4±4·7
NEM-treated RBC ($n=7$)	1·1±0·1	43·8±2·0	0·4±0·03	15·0±0·7	5·5±0·9

Animals were killed 60 min after the injection of the cells. Data are given as the mean±SE; n represents the number of animals in each group.

Table 2. Tissue distribution of unsensitized DA erythrocytes and erythrocytes sensitized with R3/13 antibody

		Percentage of the radioactivity administered				
DA erythrocytes	n	Lung	Spleen	Kidneys	Liver	Blood
Normal rats						
Unsensitized RBC						
Killed at 65 min	6	1·2±0·2	2·0±0·2	2·4±0·6	5·5±0·2	53·4±3·3
R3/13 cells						
Killed at 65 min	7	1·0±0·1	22·8±1·6	2·1±0·1	8·5±0·5	31·4±2·7
R3/13 cells						
Killed at 100 min	5	0·3±0·1	33·0±2·2	2·5±0·1	17·7±3·2	4·0±0·9
CVF-treated rats						
R3/13 cells						
Killed at 100 min	5	0·3±0·02	44·6±1·0*	2·0±0·1	4·5±0·5†	3·5±0·5

Liver and spleen uptake in CVF rats were compared with the localization of R3/13 cells in normal rats killed at 100 min: *$P<0·01$; †$P<0·02$.

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

The clearance and organ distribution of R3/13 erythrocytes

R3/13 antibody-sensitized erythrocytes were removed exponentially from the circulation of normal rats with a $T_{\frac{1}{2}}$ of 38·3±2·8 min (mean±SE, $n=21$, range 17–67 min). Their clearance in splenectomized rats was greatly decreased (Fig. 1). The clearance of R3/13-sensitized cells in cobra venom factor-treated rats was significantly longer, with a mean value for a group of nine animals being 51·7±4·2 min ($P<0·02$).

The distribution of these cells in normal and decomplemented animals was also different, there being no specific increase in the accumulation of these cells in the liver of decomplemented animals (Table 2).

The effect of complement activation on the clearance of R3/13 and NEM cells

Complement activation, initiated by the infusion (i.v.) of 5 units of CVF at an appropriate time during the erythrocyte clearance, produced a significant increase in the $T_{\frac{1}{2}}$ of both NEM- and R3/13-treated cells (Table 3). The effect of complement activation

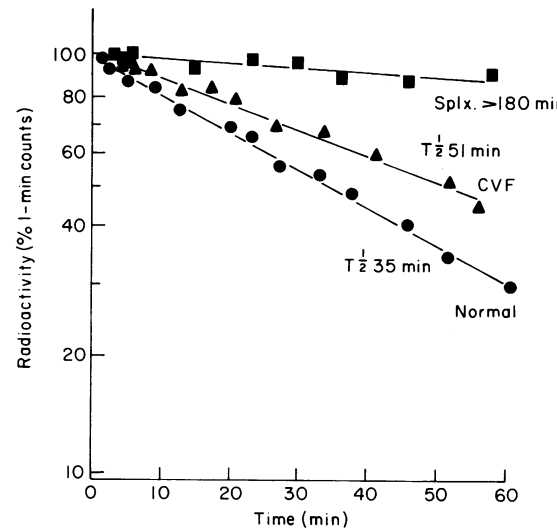

Figure 1. Clearance of R3/13 antibody-sensitized erythrocytes in a normal, cobra venom factor-treated and splenectomized rat.

Table 3. Effect of CVF on the clearance of erythrocytes sensitized with R3/13 or treated with NEM

		$T\frac{1}{2}$ of NEM cells		$T\frac{1}{2}$ of R3/13 cells	
	n	Before	After	Before	After
5 units CVF	5	27·2±2·7	41·4±3·3*	26·1±5·5	60·8±4·1†
5 units CVF (rats pretreated with Piriton)	10	18·4±2·9	21·0±1·9	21·7±3·8	59·6±5·7*
5 units heat-inactivated CVF	5	25·5±2·8	17·6±2·0†	34·6±3·7	40·9±3·0
Saline	5	18·0±1·3	11·2±0·6†	35·7±3·6	38·8±4·0

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

Table 4. Determination of blood flow using ^{86}RbCl

		Percentage cardiac output per gramme		
	n	Spleen	Kidneys	Liver
None	7	0·66±0·04	16·2±0·9	0·75±0·04
Saline	7	0·91±0·04*	13·8±0·8	0·76±0·07
5 units CVF	7	0·34±0·08*	9·2±0·9‡	0·59±0·06†
5 units heat-inactivated CVF	2	0·78, 0·82	14·8, 13·7	0·8, 0·75
5 units CVF (Piriton-pretreated rats)	4	0·69±0·1	13·0±1·5	0·8±0·04

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

on the clearance of NEM-treated cells could be reversed by pretreating the animals with 0·5 mg of Piriton (chlorpheniramine maleate). Similar results could also be obtained when the rats were pretreated with either 1 mg of Phentolamine or 1 mg of Propranolol prior to the clearance studies and the infusion of CVF (data not shown). The increase in the $T\frac{1}{2}$ of the R3/13 antibody-coated cells could not be reversed, however, by any of these three drugs, and moreover the infusion of rat serum, which had been activated *in vitro* by either zymosan or insolubilized cobra venom factor linked to Sepharose 4B, was also capable of delaying the clearance of these cells. An equal volume of normal rat serum had no such effect. During all of these studies the animals remained well and did not exhibit any of the clinical features associated with anaphylaxis.

Effects of cobra venom factor on organ blood flow

The effects of 5 units of CVF on organ blood flow were studied by its infusion (i.v.) 13 min prior to the injection of the rubidium chloride (Table 4). Although CVF produced a significant reduction in splenic blood flow, this reduction could be prevented by pretreatment with Piriton. Heat-inactivated cobra venom factor had no effect. Saline alone caused a small but significant increase in splenic blood flow.

DISCUSSION

This work was carried out in an attempt to develop methods in an experimental animal that would allow us to to investigate the factors that could influence immunospecific and non-specific red cell clearance by the spleen. The methods developed were reliable and reproducible, although we observed considerable variation in the clearance of both cell suspensions in normal animals.

The most important finding to emerge from our studies was the demonstration that complement activation *in vivo* led to a substantial reduction in splenic blood flow. The mechanisms may well involve the release of histamine, probably from mast cells activated by the complement fragments C3a and C5a, and subsequent catecholamine-induced vasoconstriction of the splenic microcirculation. When changes in blood flow were prevented using the appropriate inhibitor, no increase in the $T\frac{1}{2}$ of the NEM-treated cells was seen. It is apparent, therefore, that any studies investigating the direct effects of immune complex administration on NEM clearance should ensure that complement-initiated changes in splenic blood flow are prevented. The results obtained may otherwise be misinterpreted as reflecting a direct effect of the immune complexes on red cell clearance (Lawrence, Lockwood & Peters, 1981).

Several published reports (reviewed by Frank *et al.*, 1983) have commented on the different clearance profiles of IgG- and IgM-coated erythrocytes after their injection *in vivo*. IgM-coated erythrocytes fix complement, and during the initial phase of their clearance they are removed quickly following their binding to CR1 receptors on hepatic Kupffer cells. R3/13 antibody-sensitized cells also fix complement, but their removal from the circulation differs in several ways from erythrocytes sensitized with IgM. R3/13 cells are removed much more slowly from the blood: their predominant site of sequestration is the spleen, and once the cells have localized to the spleen they do not return to the circulation. There is, however, a small but significant uptake of R3/13 cells by the liver in normal animals that is not seen in animals treated with cobra venom factor. Specific inhibition of the clearance of these cells can be induced by *in vivo* complement activation or by the infusion of C3b fragments. The degree of inhibition achieved, however, is a modest one with no more than a three-fold increase in the $T\frac{1}{2}$. Several *in vitro* studies have shown that soluble C3b binds to complement receptors on B lymphoblastoid cell lines and polymorphonuclear leucocytes, and that it can efficiently inhibit rosette formation *in vitro* (Berger *et al.*, 1981; Eden, Bianco & Nussenzweig, 1973). We presume that C3b fragments generated *in vivo* also compete with the red cell-bound C3b for the receptors present on the splenic macrophages. This competition would interfere with the binding of these cells to the complement receptors, and would therefore delay their clearance from the circulation. This model will allow us to examine whether immune complexes can prolong the red cell clearance *in vivo* and how this is achieved.

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