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Clearance and organ localization of particles and soluble complexes in mice with circulating complexes

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Summary. The clearance and organ localization of a number of substances cleared by either Fc-dependent or -independent mechanisms was studied in normal mice and in mice with endogenously produced persistent circulating complexes. Clearance of covalent dimers of mouse IgG, chicken IgG and ovalbumin were no different between the two groups of mice. By contrast, hepatic and splenic uptake of dimeric mouse IgG (but not of chicken IgG or ovalbumin dimer) was impaired in the mice with persisting complexes. Surprisingly the rate of clearance of sheep red blood cells (SRBC) was increased in mice with persisting complexes as was hepatic uptake of polyvinyl pyrrolidone. It is suggested that the mononuclear phagocytes of mice with persistent circulating complexes are non-specifically stimulated while their ability to take up soluble complexes by Fc-dependent attachment is selectively impaired.

INTRODUCTION

The uptake of antigen–antibody complexes is mediated by cells of the mononuclear phagocyte system (MPS). It is influenced by a variety of factors including the size of the complex (Weigle, 1958; Cochrane & Hawkins, 1968), the isotype of the antibody (Mannik, Haakenstad & Arend, 1974) and its affinity (Steward, 1979; Devey & Steward, 1980). In addition, the activity of the MPS itself can influence whether the complexes are phagocytosed or not. For example, the removal from the blood of complexes larger than 11S was delayed after the injection of a large dose of soluble complexes containing human serum albumin (HSA) and anti-HSA prepared at five times antigen excess (Haakenstad & Mannik, 1974). This delayed clearance reached a maximum when a dose of complexes sufficient to produce hepatic saturation was injected. Indeed it is possible that an alteration of MPS function is responsible for the defective clearance of IgG-coated erythrocytes in patients with systemic lupus erythematosus (SLE) (Frank *et al.*, 1979). There is some doubt as to whether this effect is caused by the circulating endogenous complexes present in these individuals, because particulate complexes are taken up primarily by the spleen (Frank *et al.*, 1977) whereas soluble complexes are taken up mainly by the liver (Haakenstad & Mannik, 1976; Finbloom & Plotz, 1979a). Analogous studies in the NZB × NZW F_1 mice (which develop lupus and have circulating complexes) have also yielded equivocal results. Mannik *et al.* (1974) observed a less rapid removel of HSA anti-HSA complexes in adult NZB/W mice as compared to young controls. This was con-

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firmed by Morgan & Steward (1976) who also found a marked decrease in macrophage function in adult NZB/W mice by using a non-Fc receptor-specific measure of MPS function, the clearance of polyvinylpyrrolidone (PVP). By contrast, Morton & Siegal (1970), using colloidal carbon, found a hyperactive MPS in adult mice, and Finbloom & Plotz (1979b) found that adult NZB/W mice cleared model immune complexes faster from the serum than C3H mice.

Although the effects of one large dose of complex on the MPS have been investigated (Haakenstad & Mannik, 1974, 1976), the effects of small amounts of persistent complexes on the MPS are still uncertain. Recently, we have described the induction of endogenously produced circulating complexes in mice (Grace & Elson, 1982); because the complexes persist, such a model mimics immune complex disease more closely than those in which the animals are given one large dose of complex. The mice with persistent complexes appeared well and showed no impairment in their ability to clear soluble IgG complexes from their circulation as compared to controls (Brennan, Grace & Elson, 1982). However, when a more sensitive measure of MPS function (organ localization) was employed, it was apparent that the mice with chronic complexes had an impaired hepatic and, in some cases, splenic uptake of IgG complexes (Grace & Brennan, 1982).

The purpose of the current work is to test if this defect is due to a general malfunction of the MPS or if it is selective for complexes removed by Fc-dependent mechanisms. Accordingly, the clearance and organ localization of substances (mouse IgG dimer, chicken IgG dimer, ovalbumin dimer, SRBC and polyvinyl pyrrolidone) removed by either Fc-dependent or Fc-independent mechanisms was compared between the mice with persistent complexes and age-matched controls.

MATERIALS AND METHODS

Animals

CBA/H or CBA/H/Ig^b mice (3–6 months old) were used. CBA/H/Ig^b mice (referred to as Ig^b) are congenic with CBA/H mice except at the Ig-1 allotype locus (Micklem *et al.*, 1976; Elson, Jablonska & Taylor, 1976).

Antigens

Mouse IgG (MIgG) was prepared from normal mouse serum by precipitation with 20% sodium sulphate. The precipitate was washed in 18% sodium sulphate, redissolved and dialysed against 0·01 M HCl-Tris to pH 8·5 before elution from DEAE-Sepharose (Pharmacia) in a salt gradient of 0–0·35 M NaCl. IgG peaks were selected from the eluted fractions, pooled and purified by gel filtration on ACA 44 (LKB). Peaks of 150,000 mol. wt. were selected as monomeric mouse IgG.

Chicken gamma globulin (CGG) was prepared from normal chicken serum by precipitation with 20% sodium sulphate. The washing procedure was performed three times after which the precipitate was redissolved in distilled water and then dialysed against borate-buffered saline (BBS) at 4°.

Lyophilized ovalbumin was purchased from Sigma (grade 3).

Preparation of antibodies

Mouse anti-NAP antibodies (used to make the covalent complexes) were raised by repeated injections of NAPCAP-KLH (N(4-azido-2-nitrophenyl)-6-aminohexanoic acid-keyhole limpet haemocyanin) in Freund's complete adjuvant at a substitution ratio of nine haptenic groups per 100,000 mol. wt. protein. Anti-NAP antibodies were purified by affinity chromatography on a column of 2,4 dinitrophenyl covalently coupled to Sepharose CL4B (Pharmacia) via an ethylene diamine linking chain and eluted with 1 mM 2,4 dinitrophenol in BBS.

Preparation of covalent complexes

Dimeric mouse IgG. The method has been described in detail elsewhere (Brennan *et al.*, 1982). Briefly, the photosensitive hapten NAP was conjugated with mouse IgG at a four-fold excess of hapten. The mixture was incubated with mouse anti-NAP antibodies and then illuminated by a 60 W tungsten bulb for 15 min to photoactivate the NAP. The mixture of oligomers was separated by gel filtration on Sephacryl 300 (Pharmacia). Dimeric complexes were selected and further purified by gel filtration on the same column. Their molecular weight was estimated to be 295,000.

Dimeric chicken IgG. CGG was non-specifically cross-linked with dimethylsuberimidate (DMS) according to the method of Segal, Taurog & Metzger (1977). Ten milligrams/ml protein were covalently cross-linked in 0·2 M Tris-HCl pH 8·5 using a 30-fold molar excess of DMS. The solution was incubated for 2 hr at 30° and the oligomers separated by fractionate on a column of

Sephacryl 400 (Pharmacia). Dimeric fractions of chicken IgG were selected, pooled and reconcentrated by vacuum dialysis, then re-eluted on the same column. The molecular weight was estimated to be 300,000.

Dimeric ovalbumin. This protein was similarly cross-linked with DMS according to the method of Finbloom *et al.* (1980). Ten milligrams/ml protein were covalently cross-linked in 0·2 M Tris pH 8·5 using a 6 fold molar excess of DMS. The solution was incubated for 1 hr at 37° and the oligomers separated by fractionation on a column of ACA 34 (LKB). Dimeric fractions were again selected, reconcentrated and then rerun on the same column. The molecular weight of dimeric ovalbumin was estimated to be 92,000.

Particles

Sheep red blood cells (SRBC) were obtained from Tissue Culture Services. They were washed three times with saline prior to use.

^{125}I-PVP was obtained from the Radiochemical Centre, Amersham; (specific activity 40 μCi per mg) with a stated average molecular weight of 30–40,000.

Radiolabelling of MPS probes

Soluble complexes were iodinated using ^{125}I-Na and the solid-phase reagent 'Iodogen' (Pierce) according to the method of Fraker & Speck (1978). Free iodine was removed by passage through Agl × 2 anion exchange resin (Biorad). Unlabelled complex was added and conditions adjusted so that the final concentration of protein was 100 μg/ml containing 5×10^6 c.p.m. ^{125}I/mg protein. Each mouse was injected with 0·2 ml.

Washed SRBCs were adjusted to 50% and incubated with sodium chromate (^{57}Cr) (150 μCi) at 37° for 40 min. The erythrocytes were washed six times in PBS and made 10% in normal saline. Approximately 5×10^7 cells in 0·2 ml saline were injected into each mouse.

^{125}I-PVP was diluted with saline to 200 μg/ml and 0·1 ml was injected into each mouse.

Clearance studies

The radiolabelled probes were injected into the mice via the tail vein. After 5 min in the case of the soluble complexes, or after 30 sec in the case of the erythrocytes and PVP, and at intervals thereafter, each mouse was bled (about 50 mg per sample). The samples were weighed and the radioactivity counted so as to determine the amount of complex remaining in the circulation. The first sample taken was considered as zero time and equal to 100%. Data from each mouse was analysed individually. Whole blood clearance of the soluble aggregates was calculated as the sum of two parallel exponential decays. Values for decay half-lives for the two first-order processes and for the proportion of starting material removed at each rate (a, b) were calculated using the maximum likelihood computer program (Rothamstead experimental station). This program used the formula

$$y = a \exp(0 - 0{\cdot}693x)/t_s + b \exp(-0{\cdot}693x)t_f$$

where y = per cent remaining in whole blood and x = time (hr). SRBC clearance data was individually plotted and the $t_{\frac{1}{2}}$ value determined.

Organ localization studies

Mice were weighed and the radiolabelled probes injected into mice via the tail vein. After 5 min in experiments with soluble complexes, or after 30 sec when sheep erythrocytes or PVP had been injected, the mice were bled, the samples weighed and their radioactivity counted. From this the total (100%) number of counts injected into each mouse was calculated by assuming that their blood weight was 7·5% of their body weight. The mice were bled again just before they were killed and the percentage label remaining in their blood determined. After killing, the liver, spleen and kidneys were removed, weighed and the radioactivity in each organ counted. From this the total number of injected counts localized in each organ was calculated.

Chronic endogenous production of immune complexes

The method for producing mice with persistent complexes has been described (Grace, Elson & Coeshott, 1980). Briefly, mice of one allotype (Ig^b) were immunized against immunoglobulin from a congenic strain of mice bearing another allotype (Ig^a) (CBA/H). Spleen cells of these Ig^b mice were then transferred to CBA recipients which were challenged i.p. at weekly intervals with *B. pertussis*-Ig^a anti-pertussis. The mice were used 2 weeks after the last injection and bled prior to use. The sera were collected and tested for complexes by a complement consumption assay previously described (Grace & Elson, 1982). For these experiments age- and sex-matched CBA/H were used as controls. We have previously shown that unprimed mice injected with *B. pertussis* alone or *B. pertussis* coated with Ig^a anti-pertussis also have increased levels of circulating complexes (Grace & Elson, 1982).

Indirect haemagglutination

SRBC were washed in saline and adjusted to 1% in PBS (+0·5% heat-inactivated FCS) Sera (50 μl) were diluted serially in microtitre plates, SRBC (50 μl) added and after incubation at 20° for 30 min the cells were pelleted, the supernatant discarded and 100 μl diluted sheep anti-mouse IgG added. Haemagglutination was read at 2 hr.

RESULTS

Clearance of soluble complexes

Mice with persistent endogenous complexes were prepared and their ability to clear dimeric ovalbumin, dimeric chicken IgG and dimeric mouse IgG from the circulation compared to normal mice. For each complex the clearance rate was exponential, consisting of two parallel decay processes defined as fast half-life (t_f) and slow half-life (t_s). Table 1 summarizes the data obtained. The mean complement-consuming activity of mice with circulating complexes was significantly different from the age-matched controls in each of the three experiments. However, no difference was found between the groups in the clearance rate of any of the covalent complexes. Of interest was the fact that dimeric ovalbumin was removed from the circulation more rapidly than dimeric mouse IgG. Dimeric MIgG in turn was removed from the circulation faster in the first rapid phase than chicken IgG dimer. This was compensated to some extent as the second (slow) removal phase was longer for mouse than for chicken IgG.

Tissue uptake of soluble complexes

In our previous studies (Grace & Brennan, 1982) and in studies by other workers (Finbloom & Plotz, 1979a), blood clearance of protein complexes was not found to be sensitive enough to detect differences in the handling of soluble complexes by the MPS. Therefore tests were carried out to determine if the site of uptake rather than the rate of removal was affected in mice with endogenous complexes. The uptake of the soluble complexes in the liver, spleen and kidney in mice with endogenous complexes was compared to controls. Table 2 summarizes the data obtained 2 hours after injection of radiolabelled mouse IgG dimer, chicken IgG dimer, or dimeric ovalbumin into mice. Although there was a significant difference between the groups of mice in the complement consuming activity of the sera, there was no difference in the proportion of radiolabelled complex remaining in the blood for any of the covalent complexes. However, in the case of mouse IgG dimer, significantly less protein was detected in the livers of the animals containing complexes. (Although the mean splenic and kidney uptake of mouse IgG dimer was lower in these mice, as compared with controls, the difference was not significant.) By contrast, no difference was detected in the hepatic or splenic uptake of dimeric chicken IgG or dimeric ovalbumin between the groups. The kidney uptake of all the complexes was very similar in both groups of mice indicating that the kidney plays little or no role in the clearance of the injected complexes.

Clearance of SRBC and PVP

Again mice with persistent circulating complexes were prepared, and their ability to clear from the circulation sheep red blood cells (SRBC) and PVP was compared with controls. Table 3 summarizes the data obtained with SRBC. As before, mice injected with antibody-coated *B. pertussis* had significantly higher levels of complexes than controls. In contrast to the two-component clearance of soluble complexes, the clearance rate of ^{57}Cr-SRBC consisted of only one exponential component. This is because the clearance of SRBC is so rapid that it was not possible to obtain enough timed-point samples for accurate statistical analysis to allow breakdown of the clearance curve to more than one component. The results show a significantly faster blood clearance of ^{57}Cr-SRBC in the mice with circulating complexes compared with control mice ($P < 0{\cdot}01$).

The clearance of ^{125}I-PVP is shown in Fig. 1. The blood level falls rapidly in the first few hours, but after 4 hr there is a slower exponential fall. The rapid initial phase is probably due to extravascular loss and loss into the urine as a result of the small size of some of the PVP molecules. The fast (t_f) component and its extent can give an indication of renal damage, whereas the slow one (t_s) is believed to measure MPS uptake (Morgan & Soothill, 1975). There was no difference between control and complex mice in either the t_f or t_s values.

Tissue uptake of SRBC and PVP

The investigations were extended to see if the tissue uptake of SRBC and PVP was altered in the mice with

Table 1. Clearance of soluble aggregates

Exp. No.	Injected complex	Complex group			Control group		
		t_f (fast)	t_s (slow)	Serum complex levels (μg equivalent agg. MIgG/ml sera)	t_f (fast)	t_s (slow)	Serum complex levels (μg equivalent agg. MIgG/ml sera)
1	Mouse IgG dimer	0·75±0·20	21·8±3·4	**124±8·4	0·67±0·23	19·8±2·7	30·1±2·1
2	Chicken IgG dimer	0·91±0·50	12·2±1·4	**87±9·9	0·84±0·20	13·1±1·5	18·2±7·2
3	Dimeric ovalbumin	0·39±0·20	6·2±2·5	**86±10·3	0·33±0·20	7·5±2·6	20·3±5·0

**$P < 0·001$.

Half-life t_s (slow) and t_f (fast) values (hr) from separate experiments for dimeric complexes of mouse IgG, chicken IgG and ovalbumin in mice (four mice/group) with endogenously produced circulating complexes (Complex group) and age-matched controls (Control group). Results are mean ± 1 SD.

Table 2. Organ localization of soluble aggregates

Exp. No.	Injected complex	Group†	Serum complex levels (μg/equivalent agg. MIgG)	Blood	Liver	Spleen	Kidney
1	Mouse IgG dimer	Complex	**154±42	67±4·2	*19·3±1·8	0·46±0·06	3·2±0·86
		Control	29±16	63±4·2	23·0±0·7	0·54±0·06	4·1±0·35
2	Chicken IgG dimer	Complex	**103±15	62±2·2	12·6±0·6	0·51±0·05	3·6±0·52
		Control	21·1±6·9	64±8·0	11·4±0·8	0·47±0·05	4·4±0·48
3	Dimeric ovalbumin	Complex	*201±70	23±6·7	7·6±2·2	0·47±0·1	3·4±1·2
		Control	21±5·3	24±4·1	7·4±1·1	0·48±0·1	4·2±1·6

* $P<0·01$.
** $P<0·02$.
Four or five animals in each group.
Organ localization of covalent complexes 2 hr after injection into mice with endogenously produced circulating complexes (Complex group) and age-matched controls (Control group). Results are mean percentage uptake ± 1 SD.

Table 3. Clearance from blood of ^{57}Cr-SRBC in mice with or without endogenously produced circulating complexes

Group	*n*	Serum complex levels (μg equivalent agg. MIg/ml)	SRBC$t_{\frac{1}{2}}$ (min)
Complex	11	*30±16	**4·12±0·67
Control	14	8·2±8·0	6·79±2·77

*$P<0·05$.
**$P<0·01$.

endogenous complexes. Table 4 summarizes the results obtained. Once again there was a significant difference in the complement-consuming activity of sera between the mice containing complexes and the controls.

The proportion of radioactivity detected in the blood 2 hr after injection of ^{57}Cr-SRBC was significantly less in the mice with persistent complexes than in the controls. Similarly, less radioactivity was detected in the spleens of these mice but no difference was observed between the groups of either liver or kidney uptake. Between the groups of mice no differ-

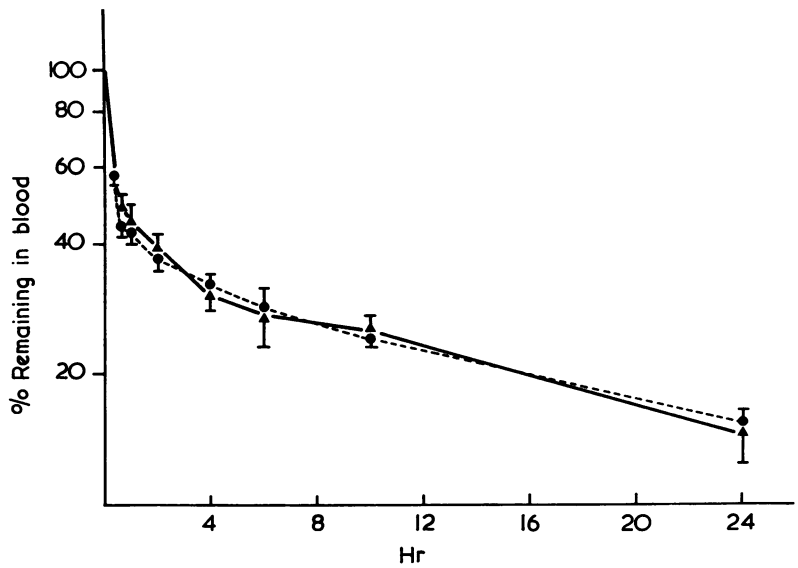

Figure 1. Whole blood clearance of ^{125}I-PVP: complex group (▲) and control group (●).

Table 4. Organ localization of SRBC ($n=4$) PVP ($n=5$) 2 hr after injection into mice with circulating complexes (Complex group) and age-matched controls (Control group) (mean percentage uptake ± SD)

Injected particle	Group	Serum complex levels (μgs equivalent agg. MIgG)	Blood	Liver	Spleen	Kidney
SRBC	Complex	***51±11	*16·7±0·9	78±5·9	**6·5±1·5	0·71±0·08
	Control	9·5±5·0	19·2±1·4	81±6·3	10·6±1·6	0·76±0·24
PVP	Complex	***84±23	15·0±23	*6·7±0·8	0·65±0·11	1·91±0·24
	Control	15·4±7·0	15·8±1·5	5·4±0·9	0·58±0·11	1·85±0·44

***$P<0·001$.
**$P<0·01$.
*$P<0·05$.

ence was detected in the proportion of radioactivity remaining in the blood 24 hr after injection of ^{125}I-PVP. However, the mice with persistent complexes had a statistically significant increase in liver uptake ($P<0·05$). There was also an increase in both spleen and kidney uptake, but this was not significant.

In all organ localization experiments, the liver, spleen and kidneys of each mouse were weighed. The only difference was a slight increase of spleen weight in the mice with persistent circulating complexes. Conversion of the spleen uptake data to percentage uptake per gram of tissue did not alter the significance of the results.

Serum antibodies to SRBC

Haemagglutination titres of sera from mice given injections of antibody-coated pertussis and controls were determined. All gave agglutination titres of less than 1, whereas sera from CBA mice immunized with SRBC gave agglutination titres in excess of 2048.

DISCUSSION

In previous reports we demonstrated that continuously circulating endogenous complexes could be induced in mice (Grace & Elson, 1982) and that the hepatic and splenic localization of trace amounts of IgG aggregates injected into these mice was reduced (Grace & Brennan, 1982). If the alterations in organ localization were purely due to a direct effect on the MPS by the agent (antibody-coated *B. pertussis*) used to induce the endogenous complexes, then similar alterations should be observed in the organ localization of other particles which are phagocytosed by Fc-independent mechanisms. It has previously been shown that the differences in organ uptake cannot be attributed to an alteration in the organ's blood supply (Grace & Brennan, 1982). The current results confirm that the hepatic and splenic localization of injected complexes known to attach to mouse Fc receptors was reduced in the mice with endogenous circulating complexes, and show that the localization of complexes cleared by Fc-independent mechanisms was unchanged.

Ovalbumin is a glycoprotein which is thought to be removed from the blood by Kupffer cells in the liver (Schlesinger *et al.*, 1978) using a recognition system specific for particular carbohydrate residues (Ashwell & Morell, 1974). As ovalbumin dimers were localized normally in the liver, it follows that the impaired hepatic uptake of IgG complexes observed in the mice with endogenous complexes is not due to a dysfunction of the Kupffer cells themselves. Rather, the current results suggest that a more selective defect is present and that it is likely to be at the level of Fc-mediated attachment. This is supported by the observation that no alterations in organ localization were detected when chicken IgG dimers were injected into the mice with circulating complexes. Chicken IgG does not bind to mouse Fc receptors (Anderson & Grey, 1974).

In human studies, the activity of the MPS has been assessed by its ability to clear particulate material from the blood, for example heat-damaged or IgG-coated erythrocytes, and it was inferred that reductions in the clearance rate of these MPS markers, such as are found in patients with SLE, are due to the endo-

genously produced circulating complexes (Frank *et al.*, 1979). In view of this, the clearance and organ localization of sheep erythrocytes and PVP was studied in mice with endogenous complexes.

Surprisingly, the clearance rate of SRBC and the hepatic localization of PVP were increased in mice with endogenous complexes. It could be argued that the faster clearance rate of SRBC resulted from the induction of anti-SRBC antibodies by the polyclonal activating effect of *B. pertussis*. This was excluded by the failure to detect anti-SRBC antibodies in mice with endogenous complexes. Alternatively, the MPS in the mice with endogenous complexes could be stimulated as a direct result of the weekly injections of antibody-coated *B. pertussis* and/or by the continual presence of the circulating complexes. The latter seems more likely as the mice were used for clearance and organ localization studies 2 weeks after the last injection of antibody-coated *B. pertussis*. In addition, antigen–antibody complexes are known to activate macrophages *in vitro* (Pestel *et al.*, 1981). Thus, the endogenous circulating complexes may non-specifically stimulate some components of the MPS without affecting others. At the same time they may specifically compete for Fc receptors on phagocytes with exogenous complexes which are dependent on Fc receptor uptake. This would explain why mice with persistent complexes have a deficiency in Fc-dependent localization, whilst their MPS appears stimulated or unaffected when tested by non-Fc-dependent probes.

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