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Clearance of soluble aggregates of human immunoglobulin G in healthy volunteers and chimpanzees

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SUMMARY

Using aggregates of IgG (AIgG) obtained by heat aggregation of a 16 g% human IgG solution, we sought a method for measuring the function of the mononuclear phagocyte system with a probe that bears more resemblance to soluble immune complexes than erythrocytes coated with anti-rhesus IgG (EIgG). It was found that intravenous administration of 10 μg AIgG/kg body weight did not cause any detectable side effects in chimpanzees. In nine healthy volunteers, a dose of 10 μg AIgG/kg body weight was used without any adverse reactions. AIgG is cleared from the human circulation with a $t_{\frac{1}{2}}$ of 26 ± 8 min (mean $\pm$ SD). The site of clearance is predominantly the liver, as shown by an average liver spleen uptake ratio of 230:100. In whole blood obtained from the volunteers, it was found that erythrocytes bound significant amounts of AIgG, suggesting that CR1 on erythrocytes is involved in the clearance of complement activating immune complexes in humans. In five of the volunteers, clearance studies with EIgG had been done in a previous study. EIgG was cleared from the circulation with a $t_{\frac{1}{2}}$ of $30 \pm 6{\cdot}2$ min (mean $\pm$ SD). The predominant site of clearance of EIgG was the spleen. These data indicate that sensitized red blood cells are cleared from the circulation differently from soluble IgG aggregates.

Keywords immunoglobulin G IgG aggregates anti-rhesus IgG volunteers chimpanzees

INTRODUCTION

Studies of mononuclear phagocyte system (MPS) function using autologous erythrocytes coated with anti-rhesus IgG have been performed in a variety of diseases (Kimberly & Ralph, 1983; Frank *et al.*, 1983). The half-life ($t_{\frac{1}{2}}$) of EIgG, radiolabelled with ^{51}Cr, in the circulation was found to correlate with the level of circulating immune complexes (CIC) and disease activity in patients with systemic lupus erythematosus (Frank *et al.*, 1979; Hamburger *et al.*, 1982). This was also observed in a longitudinal study of patients with SLE (Hamburger *et al.*, 1982), in which the $t_{\frac{1}{2}}$ of EIgG still correlated with both disease activity and the levels of CIC. Another study (Kimberly *et al.*, 1983) also found a correlation of $t_{\frac{1}{2}}$ with disease activity, but not with CIC levels. In other diseases, such as essential mixed cryoglobulinaemia (Hamburger *et al.*, 1979a), Sjögren's syndrome (Hamburger *et*

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al., 1979b), infective endocarditis (Schned *et al.*, 1983), membranous glomerulonephritis (McGinley *et al.*, 1984), cutaneous vasculitis (Dambuyant *et al.*, 1982) and rheumatoid arthritis (Fields *et al.*, 1983; Malaise *et al.*, 1985), variable results were obtained. In most cases no clear correlation was found between disease activity or C1q-binding immune complex (IC) levels and $t_{\frac{1}{2}}$ of EIgG.

After injection of radiolabelled EIgG, it has been shown (Frank *et al.*, 1983) that these sensitized cells are predominantly removed from the circulation by the spleen in both healthy volunteers and in patients with various diseases. In animal experiments (Mannik & Arend, 1971; Haakenstad & Mannik, 1974; Van Es, Daha & Kijlstra 1979; Bourne, Rayner & Verrier Jones, 1983; Veerhuis *et al.*, 1986) using preformed soluble immune complexes and antibody-coated erythrocytes, various studies have demonstrated the important role of the liver in removing these immune complexes from the circulation. From the above, it may be postulated that in human beings the lack of correlation between $t_{\frac{1}{2}}$ of EIgG and disease activity or immune complex levels, may be the result of the fact that EIgG are insoluble and predominantly cleared by the spleen, whereas in animal experiments, both soluble and insoluble IC are mostly cleared from the circulation by the liver. In this study, we describe a novel method of measuring MPS function in humans and in chimpanzees using soluble aggregates of human immunoglobulin G.

MATERIALS AND METHODS

Procurement of aggregates of immunoglobulin G (AIgG). Aggregates of immunoglobulin G were prepared by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service by heat aggregation at 63°C for 20 min of 25 ml (160 mg/ml PBS) of human immunoglobulin G. The original immunoglobulin preparation is normally used for i.m. immunoglobulin substitution in immunodeficient patients. Polyethyleneglycol 4000 (PEG) was added to the heat treated solution to obtain a final concentration of 2% PEG, followed by incubation for 1 h at room temperature (RT). After centrifugation at 5600 ***g*** at RT for 30 min, the precipitate containing the aggregates was resuspended in a 2% PEG solution in isotonic saline. This was followed by centrifugation at RT at 5600 ***g***. These last two steps were repeated twice, thereby disposing of insoluble aggregates. The final PEG precipitate was resuspended in isotonic saline to a final concentration of 1 mg protein/ml, after which the solution was filtered over a 0·45 μm filter and a 0·2 μm filter respectively.

Finally, 2 ml aliquots were transferred to 3 ml bottles and deep frozen at −70°C until used. The above steps were all performed under sterile conditions.

Characterization of aggregates of IgG. To assess the size distribution of aggregates in the AIgG preparation, 15 mg of AIgG was fractionated on a Sepharose 4B column (1·5 × 90 cm) and the protein content was determined using the Folin method. The capacity of the fractions to activate complement was tested by addition of 50 μl of a 1/5 dilution of normal human serum to 50 μl of fractions or column buffer in veronal buffer containing 1% gelatin, 5 mM $CaCl_2$ and 2·5 mM $MgCl_2$ for 15 min at 37°C, and subsequently assessed for residual complement activity using sheep erythrocytes (E) sensitized with rabbit anti E.

Radiolabelling of aggregates. On the day of study, one aliquot of AIgG was thawed, and 600–800 μg of AIgG was radiolabelled with $Na^{123}I$, by addition of approximately 37 MBq (1000 μCi) $Na^{123}I$ to the aggregate solution in a sterile tube coated with Iodogen (Fraker & Speck, 1978). The ^{123}I-AIgG-solution was then passed over a 0·22 μm Millipore filter into a sterile vial. The filter was flushed once with 3 ml saline, and an equivalent of 3·7 MBq was administered to each volunteer.

Studies in chimpanzees. We performed dose response studies with AIgG in seven adult chimpanzees (three females, four males). Studies were performed while the chimpanzees were intubated and under general anesthesia with Vetamine. After they had stabilized, 1000, 100, and 10 μg AIgG/kg body weight was injected in one, three, and three chimpanzees respectively. During these experiments, blood pressure, heart rate, and respiratory rate were registered. Blood samples were drawn for measuring haemoglobin content, haematocrit, leucocytes, thrombocytes, serum sodium, serum potassium, creatinine, total protein content, CH50, C3, C4, C1q and determination of circulating immune complexes with the C1q binding assay (Zubler *et al.*, 1976) and IgG-PEG assay (Valentijn *et al.*, 1984).

In addition, in two chimpanzees, 10 μg/kg body weight ^{123}I-labelled AIgG was injected, and disappearance of aggregates from the circulation was determined by counting residual radioactivity in serial blood samples using an LKB 1277 Gammamaster automatic gamma counter with an appropriate window setting.

Selection of volunteers. The study protocol was approved by the Ethical Committee of the Leiden University Hospital. Volunteers (22–31 years old) participated in the study after informed consent was given. They were screened for absence of disease. In order to minimalize the chance of an anaphylactic reaction occurring after infusion of AIgG, immunoglobulin A levels were determined.

Studies in healthy volunteers. Based on the results obtained in chimpanzees, a dose of 10 μg/kg body weight was chosen for the studies in healthy volunteers. Nine healthy volunteers received a mean of 680 ± 85 (SD) μg of radiolabelled (3·7 MBq) AIgG intravenously as a 3 ml bolus through a plastic cannula in the forearm, while they were supine. Radioactivity over liver, spleen, and heart were registered continuously for 1 h with a Toshiba GCA40 gamma camera connected to a nuclear medicine computer system for acquiring and processing data. Serial blood samples were obtained from the opposite forearm through a plastic cannula for 1 h to determine residual radioactivity in whole blood and plasma. Protein-bound radioactivity was measured following treatment of the samples with trichloroacetic acid with a final concentration of 10%. In addition, radioactivity associated with polymeric IgG was determined after treatment of the samples with a final concentration of 3% PEG 6000 for 60 min at 0°C. Experiments *in vitro* showed that at this PEG concentration, at least 95% of polymeric IgG and less than 5% of monomeric IgG was precipitated.

Erythrocyte bound radioactivity was assessed in aliquots of 5×10^8 erythrocytes after washing once with 5 ml PBS at 0°C. To assess the degree of complement activation after infusion of AIgG, EDTA-plasma was obtained for serial determination of C3a-levels by sandwich ELISA. In brief, dilutions of supernatants of plasma samples treated with a final concentration of 9% PEG-6000 at 4°C were pipetted into ELISA-wells coated with rabbit IgG anti-C3a, washed and subsequently interacted with rabbit IgG anti-C3a conjugated with biotin. The amount of conjugate bound was measured after interaction with streptravidin-HRP. As a standard, a C3a preparation containing 6·2 μg of C3a was used. The C3a content in this standard was determined using an Upjohn C3a-kit (kindly performed by Dr W. van Son).

Since the AIgG preparation contained a variety of aggregates of different sizes, the clearance of AIgG was studied more closely with sucrose density gradient ultracentrifugation as described by Valentijn *et al.* (1984). Centrifugation time was 90 min at 105,000 *g*. Fifteen fractions of 0·8 ml each were collected from the bottom of each gradient and assessed for ^{123}I-radioactivity. In order to detect effects of AIgG infusion on haemodynamic parameters, blood pressure and pulse rate were measured intermittently with a Dynamap® monitor during the study.

Immune complex assays. These were performed using the ^{125}I-C1q binding assay (Zubler *et al.*, 1976), and the results expressed in μg equivalent of aggregated IgG as described by Kauffmann, Van Es & Daha (1979). Determination of IgG-containing immune complexes was performed using the IgG-PEG assay as described by Valentijn *et al.* (1984).

Statistical analyses. These were performed using Student's *t*-test for unpaired samples and linear regression analysis. Analysis of blood curves was performed using linear regression analysis.

Clearance of erythrocytes coated with anti-rhesus IgG. In five of the volunteers, clearance studies of IgG-coated erythrocytes (EIgG) were performed as described by Lobatto *et al.* (1987).

RESULTS

The protein profile obtained after fractionation of AIgG on Sephadex 4B (1·5 × 90 cm: see Fig. 1) indicates that the AIgG preparation consists of ± 60% aggregated material with a size ranging from 300 to 20,000 kD and that ± 40% monomeric IgG is present as well. Analyses of AIgG preparations kept at −70°C for 3, 6, 12, and 18 months were performed, revealing that size distribution remained constant. Fractions were also tested for complement consumption, showing that fractions containing AIgG caused complement consumption (CH50), while this could not be detected in fractions containing monomeric IgG.

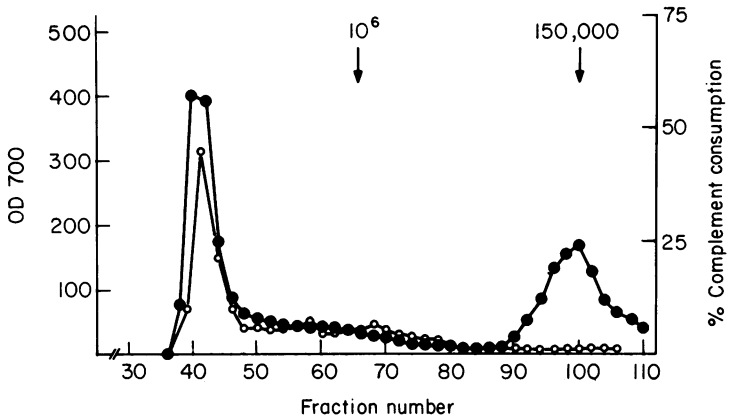

Fig. 1. Gelfiltration on a Sepharose 4B column of 15 mg AIgG. Protein profile (●) and complement consuming activity (○) are shown.

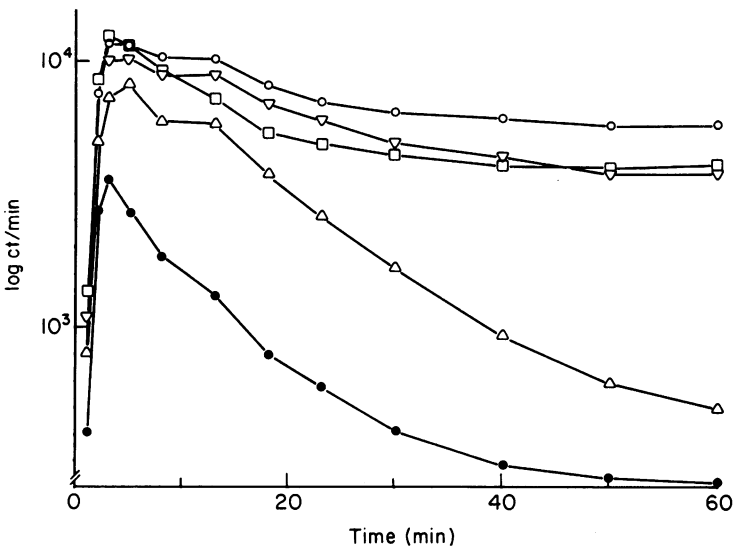

Fig. 2. Disappearance of radioactivity from the circulation in one volunteer after ^{123}I-AIgG i.v. as measured in whole blood (□), plasma (○), PEG 3% precipitable radioactivity in plasma (△), trichloroacetic acid 10% precipitable radioactivity (▽), and erythrocyte bound radioactivity (●).

Chimpanzee studies. Infusion of 1000 μg AIgG/kg body weight (50 mg) resulted in a dramatic fall in BP during the first 5 min after infusion, after which BP returned to normal. Infusion of 100 μg/kg or 10 μg/kg body weight had no detectable effect on BP. After a 1000 μg/kg body weight infusion of AIgG, few leukocytes were detectable in the circulation for 10 min after infusion. One hour after AIgG infusion, the WBC count was 43% above baseline. After injection of 100 μg AIgG/kg body weight, there was still a 60% drop in WBC count, rising to supranormal values at 1 h after injection. After infusion of 10 μg AIgG/kg body weight, the WBC count fell by less than 5%.

Using radiolabelled AIgG in two chimpanzees, the $t_{\frac{1}{2}}$ in whole blood was found to have a rapid rate of 3·2 min and a slow rate of 320 min.

Signs of systemic complement activation were not detectable in any of these chimpanzees, as measured by determination of the CH50 or C3, C4 and C1q levels. The C3a-levels were not

Table 1. C3a levels before and at various time intervals after infusion of AIgG in healthy volunteers

Time (min)	Mean C3a ng/ml (±SD)	Mean % change related to base level
0	115·5 (68·8)	
1	139·5 (77·4)	20·8
2	172·5 (120·2)	49·3
3	206·9 (148·6)	79·1
5	186·1 (133·4)	61·1
8	202·7 (165·3)	75·5
30	143·5 (115·6)	24·2
60	129·7 (93·6)	12·2

measured. Immune complexes were not detectable in the C1q-binding assay or the IgG-PEG assay before or after infusion of AIgG.

Studies in human volunteers. Based on the results of the primate experiments, an AIgG dose of 10 μg/kg body weight was chosen for administration to human volunteers. Nine volunteers were given ^{123}I-AIgG intravenously. No changes in blood pressure or pulse rate were detected during, or within 1 h after the infusion in any of the volunteers. None had any complaints during the study.

The disappearance curves of radioactivity in one volunteer are shown in Fig. 2. Radioactivity precipitable in 10% trichloroacetic acid (TCA) to measure protein-bound radioactivity, had a mean initial value of 89% ± 5% (SD) of radioactivity in plasma. Over 1 h, TCA precipitable radioactivity decreased to a mean of 78% ± 11·3% (SD), indicating degradation of AIgG with release of ^{123}I into the circulation. The $t_{\frac{1}{2}}$ of PEG 3% precipitable radioactivity had a mean rapid rate of 4·6 ± 1·7 min (SD) and a slow rate of 26 ± 8 min. The percentage of erythrocyte bound radioactivity compared to whole blood was uniformly highest at 1 min after injection of AIgG, with a mean of 24·3% ± 20·5 (SD), gradually decreasing to 4·3% ± 1·3 (mean ± SD) after 1 h.

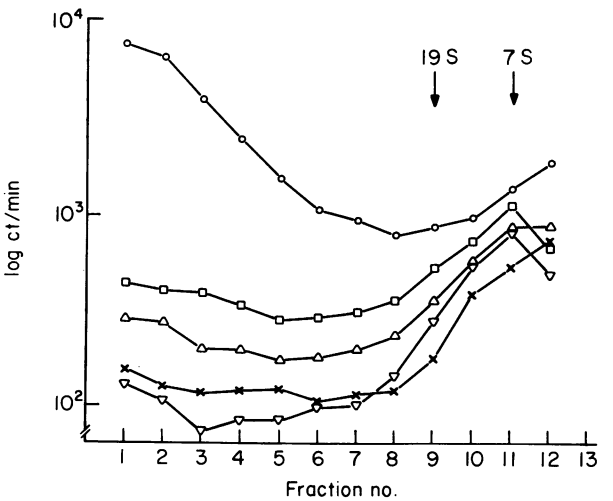

Fig. 3. Sucrose density gradient analyses of plasma samples obtained at 1 (○), 3 (□), 5 (△), 8 (×) and 13 (▽) min after i.v. injection of ^{123}I-AIgG in a volunteer. Larger aggregates disappear most rapidly as indicated by the rapid decrease in radioactivity in the fractions with lower numbers.

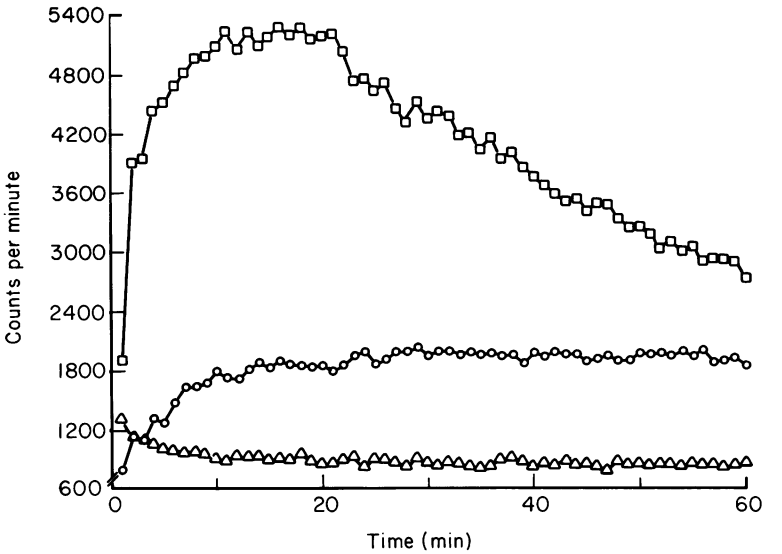

Fig. 4. Continuous monitoring of radioactivity over liver (□), spleen (○) and heart (△) over 1 hour after i.v. injection of ^{123}I-AIgG in a healthy volunteer.

After infusion of AIgG, no changes in WBC count, CH50, C3, C4 or C1q levels were seen in any of the volunteers and no circulating immune complexes were demonstrated in the C1q-binding assay or IgG-PEG assay either before or after administration of AIgG.

An increase in C3a levels was detectable in all volunteers within a few minutes after injection of AIgG. The time elapsed until the peak value of C3a was reached was 3–8 min. Table 1 shows C3a levels before, and at various times after infusion of AIgG.

In Fig. 3. a representative ultracentrifuge profile of plasma samples drawn at various times after infusion of AIgG in one of the volunteers is shown. The large aggregates are shown to be removed most rapidly from the circulation, whereas small aggregates and monomeric IgG remain in the circulation at 1 h after injection of AIgG.

The liver and spleen uptake of AIgG had a uniform pattern in all volunteers. Figure 4 shows an example of radioactivity over liver, spleen, and heart during 1 h in one volunteer, with a peak of

Table 2. Liver/spleen uptake ratio for EIgG and AIgG in volunteers

	Ratios*		
	EIgG	AIgG	
Volunteer	(at 60 min)	(at 15 min)	(at 60 min)
1	3/100	187/100	86/100
2	8/100	232/100	225/100
3	16/100	203/100	166/100
4	15/100	332/100	189/100
5	4/100	114/100	79/100

* Ratios are expressed as uptake of radioactivity in the liver divided by the splenic uptake, which has arbitrarily been set at 100.

hepatic uptake of ^{123}I-AIgG at 15 min after injection, and a gradual decrease in hepatic activity until 1 h. Splenic uptake lags behind, and activity decreases more slowly than in the liver. The curve of radioactivity detected over the heart region shows residual activity in the circulation at various points. The maximum ratio of liver/spleen uptake (LS ratio), is $230 \pm 73/100$ (mean $\pm$ SD).

In five of the volunteers, clearance studies were performed with both EIgG and AIgG, although not at the same time. Clearance rates as expressed by $t_{\frac{1}{2}}$ were similar for both probes (Lobatto *et al.*, 1987). The site of removal from the circulation is quite different, as shown in Table 2. While the L/S uptake ratio for EIgG is 11/100 at 1 h after injection, this ratio is 180/100 at the same point in time for AIgG. From this, it may be concluded that while the site of clearance of EIgG is predominantly the spleen, it is predominantly the liver for AIgG.

DISCUSSION

In this study we have shown that infusion of aggregates of human immunoglobulin G can be performed safely in animals and humans, and is useful in determining the capacity of the MPS to remove soluble immune complexes from the circulation. In previous studies from this laboratory (Van Es *et al.*, 1979; Veerhuis *et al.*, 1986), AIgG was infused into rats to determine the uptake of aggregates in various organs. Over 90% of AIgG injected was detected in the liver, and could be shown to be located in sinusoids, presumably Kupffer cells. In safety studies carried out in chimpanzees, it was found that a dose of 1000 μg/kg body weight of AIgG causes profound shock, respiratory distress, and extreme leucocytopenia. This is compatible with complement activation *in vivo* by AIgG, resulting in sequestration of leucocytes in the pulmonary circulation, by a process similar to that described in haemodialysis patients (Craddock *et al.*, 1979).

After 100-fold reduction of the dose, we found that at 10 μg AIgG/kg body weight, no systemic effects could be demonstrated in chimpanzees. With the same dose, no side effects were noted in healthy volunteers either, although C3a, signifying complement activation, was generated in all of them. On average, the C3a levels increased by 80% compared to baseline levels, which is much less than the increase found in haemodialysis patients. In a recent study (Hakim *et al.*, 1984), it was shown that a rise in C3a levels could be found in all dialysis patients studied; however only patients with the so-called 'first use' syndrome had a large increase in C3a levels. In asymptomatic dialysis patients, a 4-fold increase in C3a levels was noted, while in patients with the 'first use' syndrome, a 13-fold increase in C3a levels was observed (Hakim *et al.*, 1984). In patients with serum sickness after injection of antithymocyte globulin, a significant rise in C3a levels was also found (Lawley *et al.*, 1984). In our study, there appears to be sufficient complement activation to allow clearance of AIgG by complement receptors, but insufficient complement activation to cause detectable side effects.

Using EIgG as a probe of MPS-function, most studies (Kimberly & Ralph, 1983; Frank *et al.*, 1983) have failed to produce results showing a correlation between clearance rates of EIgG from the circulation, and disease activity or CIC levels. In order to investigate possible differences in clearance mechanisms between EIgG and AIgG, studies with both probes were perfomed in five of the volunteers, although not simultaneously. We found that while EIgG was mainly cleared from the circulation by the spleen, AIgG was mainly removed by the liver (Table 2). The $t_{\frac{1}{2}}$ of clearance from the circulation was similar for both probes.

In a mouse model, it was recently shown that clearance of human AIgG from the circulation could be slowed by the presence of immune complexes in the circulation, probably by decreased hepatic uptake of AIgG (Jimenez & Mannik, 1982). With the method used in our study, we feel that it is safe to use human AIgG as a probe of MPS function in patients with immune complex diseases.

Another finding in this study that merits attention is the role of binding of AIgG to erythrocytes as an instrument in the removal of AIgG from the circulation. The CR1 receptor on erythrocytes in non-human primates plays a role in the removal of complement-activating preformed IC from the circulation (Cornacoff *et al.*, 1983). From our study, two mechanisms of transport of AIgG to the MPS can be deduced, one way consisting of direct transportation of AIgG to Kupffer cells and splenic phagocytes, where they can bind to Fc receptors and CR1 receptors after complement

activation. The second route could consist of the binding of AIgG bearing C3b to CR1 receptors on erythrocytes followed by transport to liver and spleen for further processing. A question arising from this study is whether a decreased number of CR1 receptors on erythrocytes, such as can be found in SLE (Iida, Monaghi & Nussenzweig, 1982; Walport *et al.*, 1985; Wilson *et al.*, 1982), will influence the clearance of AIgG from the circulation and the uptake of AIgG by the MPS.

Studies on clearance of AIgG in patients with SLE and rheumatoid arthritis are now in progress. We suggest that studies of MPS function can be carried out safely with AIgG at the dosage employed in this study. In patients with a complete IgA deficiency, anti-IgA antibodies may be present, leading to potential anaphylactic reactions after the administration of AIgG (Vyas, Perkins & Fudenberg, 1968), so in our opinion these patients should be excluded from studies using AIgG. A significant advantage of the method reported in this study is that AIgG possesses many biological activities similar to immune complexes. AIgG can fix complement (Christian, 1960), induce an Arthus reaction (Ishizaka, 1963), and is cleared from the circulation of mice (Brown *et al.*, 1973) and rats (Mauer *et al.*, 1972) by the MPS in a manner similar to immune complexes, so providing a greater chance of finding a correlation with disease activity or CIC levels than EIgG. An additional advantage over EIgG is that rhesus negative patients can also be studied with AIgG.

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