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Key role of macrophages in hypotensive side effects of immunoglobulin preparations. Studies in an animal model

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SUMMARY

Intravenous administration of certain immunoglobulin preparations may cause severe adverse reactions, especially in hypogammaglobulinaemic patients. Because the exact mechanism of the adverse reactions is still unknown, we investigated the severe, prolonged hypotension induced in anaesthetized rats on rapid i.v. infusion of standard immunoglobulin preparations. The hypotensive response was previously shown to be associated with IgG aggregates in the preparations but independent of complement activation. We found that the hypotension could be prevented by treating the rats with a specific receptor antagonist of platelet-activating factor; or by depletion of the macrophages of the rats; or by pretreatment with monomeric IgG. This provided evidence that the hypotension is initiated by interaction of IgG-aggregates with Fc-receptors on macrophages, leading to the production of platelet-activating factor. We conclude that the rat model provides a sensitive and reproducible test system for macrophage-activating properties of immunoglobulin preparations for i.v. administration which may lead to vasoactive side effects.

Keywords immunoglobulins side effects macrophages

INTRODUCTION

In the 1950s i.v. administration of standard immunoglobulin preparations (SIg), prepared with Cohn's cold ethanol fractionation, caused severe anaphylactoid adverse reactions, especially in immunodeficient patients. The reported reactions, ranging from facial flushing, nausea and fever to dyspnea, hypotension, and circulatory shock, are generally assumed to be related to the presence of IgG aggregates, that may spontaneously activate the complement system of the recipient (Barandun & Isliker 1986). For this reason, SIg has to be administered intramuscularly. In the past 20 years, several modified immunoglobulin preparations that can be intravenously infused in a large dose have been developed. Their safety with regard to vasoactive side effects is generally tested by *in vitro* measurements such as the aggregate content, the so-called anti-complementary activity (Nydegger 1985; Barandun & Isliker, 1986), and the prekallikrein activator activity (Alving *et al.*, 1989).

In a previous report (Bleeker *et al.*, 1987) we described an additional safety test in an animal model. It was found that i.v. administration of SIg to rats caused prolonged hypotension, but a modified i.v. immunoglobulin preparation administered with well-established safety to patients, did not. This hypotensive response appeared to be associated with IgG aggregates but could not be predicted from *in vitro* measurement of the anti-complementary activity. Experiments with complement depletion of the rats with Cobra venom factor provided further evidence that the vasoactive reaction was no based on complement activation. As has also been observed by Medgyesi *et al.* (1988), the prolonged hypotension was not related to prekallikrein activator either. Prekallikrein activator may be present in high concentrations in certain immunoglobulin preparations and may induce a transient hypotensive response of several minutes after sensitizing the rats to bradykinin (Bleeker *et al.*, 1982).

To substantiate the value of the detection system of vasoactive side effects in rats as part of the quality control of immunoglobulin preparations intended for i.v. administration, we directed the present study toward the mechanism of the hypotension. Since IgG aggregates may also interact with Fc-receptors on macrophages, we investigated the possible involvement of macrophages in the hypotensive response in the rats. In the majority of our experiments, rats received an i.v. infusion of SIg (for i.m. administration), because the clinical side effects have been documented primarily for these immunoglobulin preparations.

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MATERIALS AND METHODS

Animal model

Female Wistar rats weighing 200–250 g were anaesthetized by

i.p. injection of pentobarbital, 50 mg/kg body weight. A polyethelene cannula filled with heparin (50 U/ml) in physiological saline was inserted into the left carotid artery for measurement of blood pressure and for taking blood samples. The mean arterial blood pressure, the pulse pressure and the heart rate were derived electronically from the signal of a pressure transducer and were continuously recorded. The stability of the blood pressure was checked for 10 min; rats with unstable blood pressure or a pressure <100 mmHg were excluded from the studies. Immunoglobulins and drugs were administered intravenously into a lateral tail vein at a rate of 0·1 ml/sec.

Human serum albumin (HSA) was produced by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam). Platelet-activating factor (PAF) was obtained from Sigma Chemical Co (St Louis, MO) as a 2 mg/ml stock solution in chloroform; it was diluted in phosphate-buffered saline (PBS), pH 7·4, containing 0·25 g of HSA per 100 ml. The PAF-receptor antagonist L-652,731 was a generous gift of Dr J. C. Chabala (Merck Sharp and Dome, Rahway, NJ). L-652,731 was first dissolved in dimethyl sulphoxide (DMSO, 2 mg/ml) and was 100-fold diluted in PBS immediately before infusion.

Elimination of macrophages

In three rats the macrophages of liver and spleen were eliminated by i.v. administration of liposome-encapsulated dichloromethylene diphosphonate (DMDP) 3 days before the experiment. Details of the technique, including the preparation of the liposomes, have been described earlier (Van Rooijen & Claasen, 1988). Briefly, 150 mg of phosphatidylcholine and 22 mg of cholesterol were dissolved in chloroform in a round-bottomed flask. The thin film that formed on the interior of the flask after low-vacuum rotary evaporation at 37°C was dispersed by gentle rotation for 10 min in 20 ml of PBS containing 3·8 g of DMDP. The liposomes were centrifuged twice in PBS at 100 000 ***g*** for 30 min to remove free, non-encapsulated DMDP. Thereafter, the liposomes were resuspended in 8 ml of PBS, and 2 ml were intravenously injected in each of the rats. DMDP was a kind gift of Proctor and Gamble (Cincinnati, OH). The elimination of the macrophages was checked after the experiments by an immunoenzyme histochemical method with the monoclonal antibodies ED2 and ED4 to identify the macrophages (Dijkstra *et al.*, 1985).

Analysis of blood samples

Blood samples (0·25 ml in 0·01 ml of 50 mM EDTA) were collected from the arterial cannula for blood cell counting and determination of the haemolytic complement. Red blood cells, leukocytes and platelets were counted with an electronic cell counter (Coulter Electronics, model ZF, Dunstable, UK). The leukocyte differentiation was determined microscopically in Giemsa-stained blood smears. The results were expressed as a percentage of the control value before the administration of the test solution. The haemolytic complement was determined from the lysis of sensitized sheep red blood cells, added to serially diluted, recalcified plasma samples, as described earlier (Bleeker *et al.*, 1987).

Immunoglobulins

Two commercially available immunoglobulin preparations produced by the CLB were used in the experiments. A SIg preparation for i.m. administration is prepared from a pool of human plasma by a modification of the Cohn ethanol fractionation technique (Brummelhuis, 1983). A preparation of immunoglobulin suitable for i.v. administration (i.v.Ig) is produced by pH 4 treatment of Cohn fraction II with the addition of traces of pepsin; this preparation is provided in lyophilized form.

For the present study two modified preparations were used: (i) an i.v.Ig (6 g/100 ml) was aggregated by heating for 60 min at 57°C and subsequently filtered through a 0·2-μm filter unit; and (ii) a SIg was fractionated by gel chromatography on a $2{\cdot}5 \times 90$ cm column filled with Ultrogel ACA34 (LKB, Bromma, Sweden); 750 mg of the SIg were applied to the column in a buffer containing 0·066 M Tris-HCl (pH 7·4) and 0·5 M NaCl; the flow rate was 0·5 ml/min. Column fractions comprising the IgG monomer peak were pooled and concentrated by dialysis against PBS containing 15% polyethylene glycol (mol. wt 20 kD).

Characterization of immunoglobulins

Protein concentrations were measured photometrically at 280 nm, using an absorbance coefficient of 14·7 cm^{-1} for a 1 g/100 ml solution. The mol. wt distribution was determined with gel chromatography as described above. The anti-complementary activity was determined in a haemolytic assay with sensitized sheep blood cells and guinea pig serum as the source of complement, as described earlier (Bleeker *et al.*, 1987). The anti-complementary activity was expressed as the amount of inactivated complement per mg of immunoglobulin (CI50/mg). Prekallikrein activator activity, measured with a chromogenic assay as described earlier (Bleeker *et al.*, 1987), ranged from 0·1 to 1·2 U/g in SIg and was $<0{\cdot}01$ U/g in i.v.Ig (a sample with 100 U/*l* corresponds to the 100% FDA standard for prekallikrein activator).

RESULTS

Hypotensive reactions in the rat

Figure 1 shows the comparison of the effects of rapid i.v. administration of an arbitrarily chosen SIg, an i.v.Ig and a heat-aggregated i.v. immunoglobulin preparation (HAIg). The *in vitro* characteristics of these preparations are summarized in Table 1. SIg had a relatively high dimer content, whereas HAIg contained mainly larger polymers and showed a very strong *in vitro* complement consumption. In the rat, SIg infusion induced severe hypotension; the blood pressure started to fall after a latent period of about 1 min and reached its lowest level after about 15 min. Recovery of the blood pressure did not occur before 1–3 h after administration. The hypotension was accompanied by a decrease in haemolytic complement. Infusion of i.v.Ig gave no hypotension and only minimal decrease in haemolytic complement. HAIg induced less hypotension than did SIg, but a much stronger decrease in CH50 titre, to below the detection level of our assay. The decrease in blood pressure and CH50 titre was accompanied by changes in the peripheral blood cell count; a biphasic reaction in the number of circulating neutrophilic granulocytes and a transient thrombocytopenia,

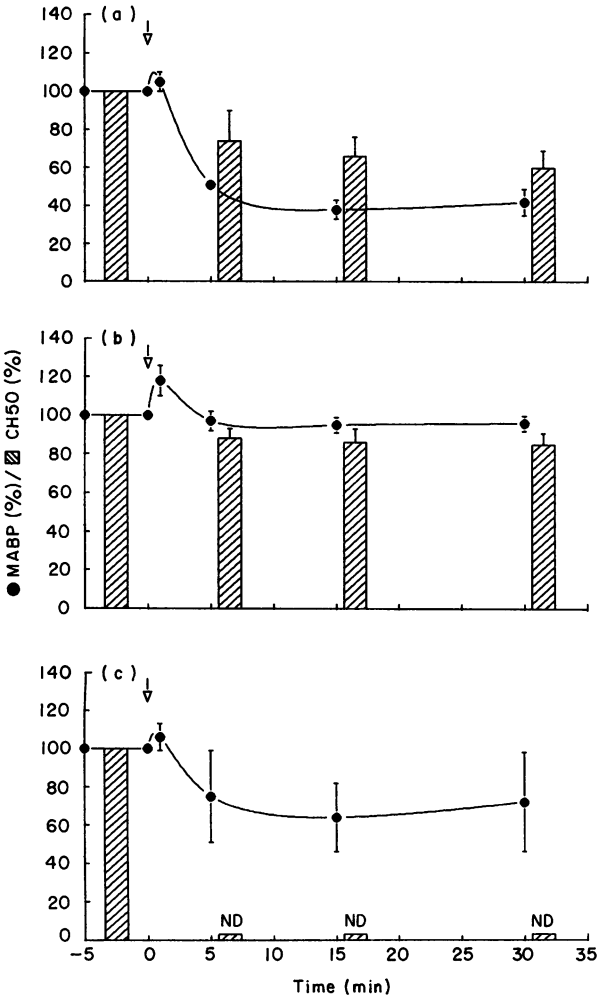

Fig. 1. Changes in percentage of mean arterial blood pressure (MABP) (●) and haemolytic complement (CH50) (■) in rats after i.v. administration of standard immunoglobulin (SIg) (a); i.v. immunoglobulin (i.v.Ig) (b); and heat-aggregated i.v.Ig (HAIg) (c). Each preparation was given to three rats at a dose of 250 mg/kg body weight. The results are presented as percentage of the control value (mean ± s.d.). ND = not detectable.

with minimal values after 5 min. Table 1 shows the changes after 5 min. After HAIg infusion, neutrophils were virtually absent in the blood after 5 min, but after 15 min an overshoot was found to about 200%. i.v.Ig gave negligible changes in the blood cell count. Furthermore, after 15 min a marked increase in red blood cell count was observed after SIg, which was interpreted as a decrease in plasma volume.

PAF-receptor antagonist L-652,731

In a first series of experiments the efficacy of L-652,731 treatment towards the effect of an i.v. bolus dose of PAF was tested. In three control rats a single dose of 100 ng of PAF induced an instantaneous hypotensive reaction with a duration of about 5 min, with a maximum decrease of 57 ± 14% after 1 min. Three other rats received 0·4 mg of L-652,731 together with the PAF, followed by continuous L-652,731 infusion at 0·13 mg/min (the control rats received an equivalent volume of a buffer contain-

Table 1. *In vitro* characteristics and *in vivo* effects of SIg, i.v.Ig and HAIg

	SIg	i.v.Ig	HAIg
In vitro			
Anti-complementary activity (CI50/mg)	5	0·02	100
IgG dimers (%)	18	7	6
IgG polymers (%)	5	3	13
In vivo			
Neutrophils (5 min)	−74 ± 14	−10 ± 38	−100
Platelets (5 min)	−32 ± 4	−10 ± 13	−50 ± 4
Plasma volume (15 min)	−22 ± 20	−6 ± 8	+4 ± 2.4

The values are the percentage change, mean ± s.d. ($n = 3$).

* Used in the experiments of Fig. 1. The *in vivo* effects relate to the same experiments as Fig. 1.

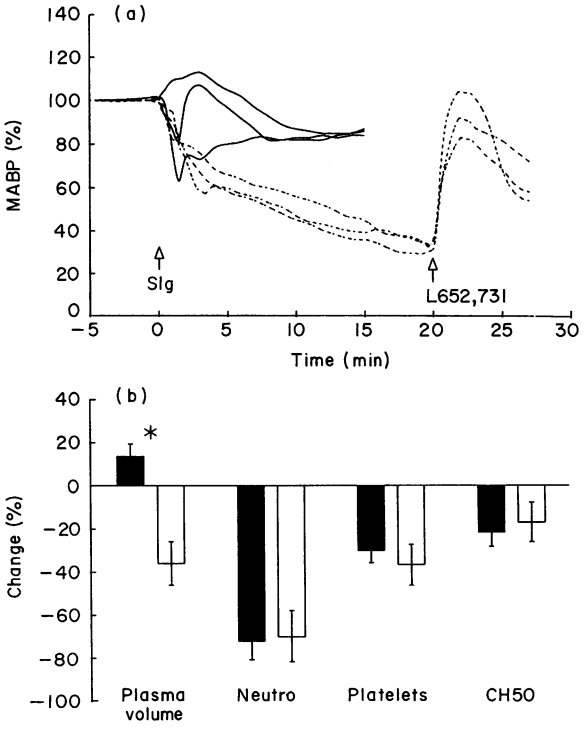

Fig. 2. Effect of treatment with the PAF-receptor antagonist L-652,731 on the reactions induced by i.v. infusion of 250 mg SIg/kg body weight. The SIg was tested in three antagonist-treated and three control rats. (a) Blood pressure recordings of the individual rats (—), scaled as a percentage of the control value. The control rats (– – –) received a bolus dose of L-652,731, 20 min after the SIg. (b) Percentage changes in plasma volume (15 min after SIg), neutrophil count (5 min after SIg), platelet count (5 min after SIg) and haemolytic complement (CH50, 15 min after SIg) in treated (■) and in control (□) rats. The results are mean ± s.d.

* Significant difference (student's t-test, $P < 0.05$).

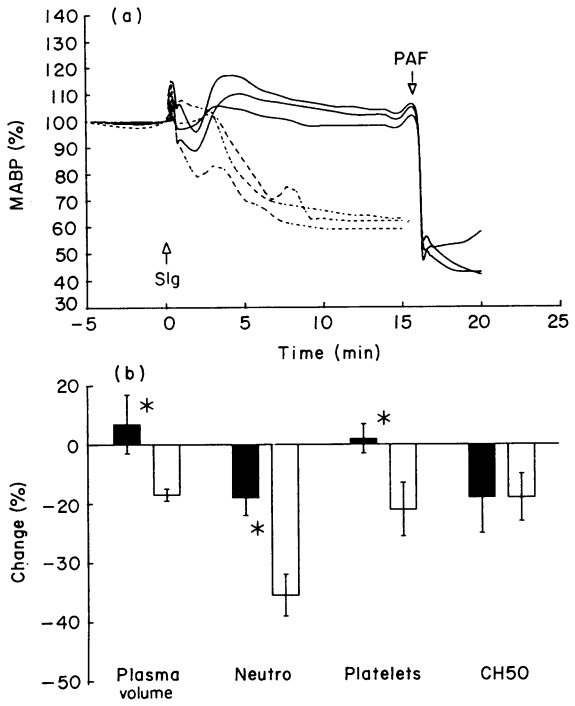

Fig. 3. Effect of macrophage depletion of rats by pretreatment with DMDP-containing liposomes on the reactions induced by i.v. infusion of 250 mg SIg/kg body weight. The SIg was tested in three DMDP-treated (—) and three control (– – –) rats. (a) Blood pressure recordings of the individual rats, scaled as a percentage of the control value. The DMDP- pretreated rats received a bolus dose of PAF, 16 min after the SIg. (b) Percentage changes (DMDP-treated, ■ and control, □ rats) in plasma volume (15 min after SIg), neutrophil count (5 min after SIg), platelet count (5 min after SIg) and haemolytic complement (CH50, 15 min after SIg). The results are mean ± s.d. * Significant difference (student's *t*-test, $P < 0.05$).

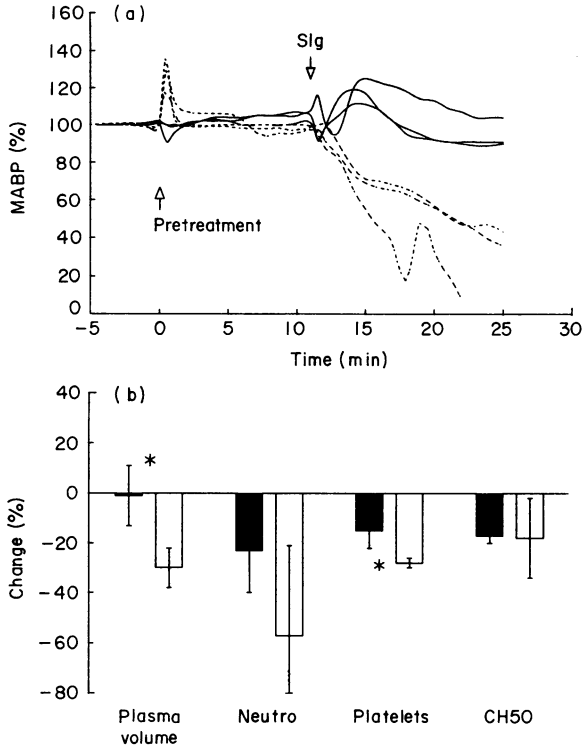

Fig. 4. Effect of pretreatment of rats with 250 mg monomeric IgG/kg body weight on the reactions induced by i.v. infusion of 250 mg of SIg/kg body weight. Pretreatment was at time zero. Control rats received 250 mg HSA/kg body weight. SIg was administered 11 min after the pretreatment. (a) Blood pressure recordings of the individual rats, treated (—) and control (- - -) scaled as a percentage of the control value. (b) Percentage changes in plasma volume (15 min after SIg), neutrophil count (5 min after SIg), platelet count (5 min after SIg) and haemolytic complement (CH50, 15 min after SIg) in pretreated (■) and control (□) rats. The results are mean ± s.d. * Significant difference (student's *t*-test, $P < 0.05$).

ing 1% DMSO). In this case a significantly lower fall in blood pressure was observed: 29 ± 13% (Student's *t*-test, $P < 0.05$). The single dose of PAF produced only minimal changes in blood cell count and haemolytic complement.

Figure 2 shows that treatment with L-652,731 produced a considerable reduction in the hypotensive reaction on SIg administration, especially in the hypotension after 15 min. Furthermore, it was found that a single dose of L-652,731 given to the control rats during the hypotension caused a temporary return of the blood pressure to normal level. The fall in plasma volume was also abolished by the PAF receptor antagonist, but the decrease in the number of circulating neutrophils, platelets and CH50 was unchanged.

In vivo *elimination of macrophages*

Immunoenzyme histochemistry: in the spleen of normal rats, ED2-positive macrophages populated the red pulp and branched to ED3-positive macrophages in the marginal zone and in the periphery of the white pulp. The macrophage population in the liver of normal rats consisted of ED2-positive Kupffer cells along the sinuses. After pretreatment with DMDP liposomes, these ED2-positive and ED3-positive macrophages had disappeared completely from spleen and liver. The DMDP-liposome treated rats appeared to be in good condition and had normal blood pressure under anaesthesia. The treatment caused no changes in neutrophil, monocyte, platelet or erythrocyte count in the blood.

Figure 3 shows the effect of DMDP-mediated elimination of the macrophages on the responses to SIg administration (250 mg/kg body weight); Fig. 3a shows that the typical prolonged hypotension was completely prevented; only some minor changes in the first 5 min were observed. Intravenous administration of PAF (500 ng/kg body weight) 16 min after the SIg resulted in 50% decrease in blood pressure, indicating that the prevention of hypotension was not due to insensitivity to PAF. Three untreated control rats received the same SIg and showed the usual decrease in blood pressure. The decrease in plasma volume, calculated from the increase in red blood cell count, and the fall in platelet count after 5 min were also prevented. The decrease in number of circulating neutrophilis was diminished. However, the decrease in CH50 titre remained unchanged compared with the control rats.

Pretreatment with monomeric IgG

IgG monomers were isolated with gel chromatography from an SIg preparation containing 20% dimers and 5% polymers. The

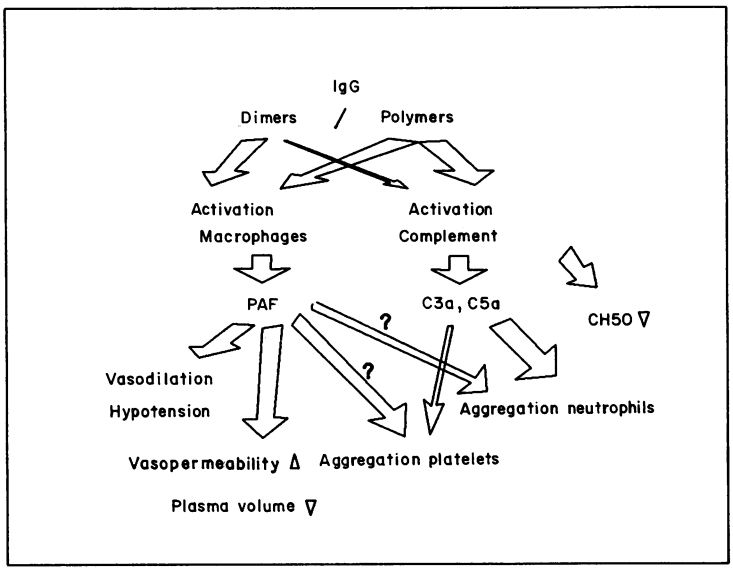

Fig. 5. Schematic diagram of the hypothetical sequence of events induced by IgG aggregates in immunoglobulin preparations.

isolated monomer fraction contained 4·5% dimers and no polymers. Three rats were pretreated intravenously with the monomer fraction 11 min before the administration of SIg. Three control rats received HSA instead of IgG monomers. Figure 4 shows that the pretreatment itself caused only minimal changes in blood pressure. A blood sample taken 10 min after the administration of either IgG monomers or HSA showed an increase of about 50% in the number of circulating neutrophils, but no significant changes in erythrocyte count, platelet count or CH50 titre. Figure 4 shows that the hypotensive reaction and the decrease in plasma volume on SIg administration were completely prevented by the pretreatment with IgG. A small but significant reduction in the thrombocytopenia was observed. However, the changes in neutrophil count and CH50 titre were not significantly diminished.

In another series with three rats, pretreatment with i.v.Ig instead of IgG monomer fraction SIg also prevented the hypotensive reaction upon subsequent SIg administration.

DISCUSSION

The present study was focused on the mechanism of the prolonged hypotensive response induced by SIg in pentobarbital-anaesthetized rats. Previously, we had shown the hypotensive factor to be associated with IgG dimers and polymers (Bleeker *et al.*, 1987). Since IgG aggregates may activate the complement system in a similar way to immune complexes, we first investigated complement activation as a cause of the hypotension. The hypotension was indeed accompanied by complement activation, as evidenced by a decrease in the haemolytic complement titre. However, we observed no decrease in hypotensive response after depletion of the complement system by pretreatment of the rats with Cobra venom factor (Bleeker *et al.*, 1987). Furthermore, activation of the complement system by i.v. administration of Cobra venom factor itself was not accompanied by a fall in blood pressure. Analysis of the ratio between the decrease in blood pressure and in haemolytic complement revealed differences between different IgG aggregates. Figure 1 shows that SIg, which contains mainly IgG dimers, induced a strong hypotensive response with a minimal decrease in complement, whereas HAIgG, containing larger aggregates, induced mainly a decrease in complement. This led to the conclusion that complement activation and hypotension are independent phenomena. Therefore, another mediator of the hypotensive response had to be considered.

In recent years, several investigators have provided evidence that *in vivo* reactions to immune complexes or HAIgG are linked by PAF generation (Camussi et al., 1981; Inarrea, Alonso & Sanchez-Crespo, 1983; Sanchez-Crespo *et al.*, 1985; Doebber, Wu & Biftu, 1986). Moreover, i.v. infusion of PAF may provoke the same responses as infusion of immune complexes, i.e. neutropenia, thrombocytopenia, increased vascular permeability and hypotension. Doebber, Wu & Biftu (1986) used L-652,731, a potent PAF receptor antagonist with a high degree of specificity (Hwang *et al.*, 1985), to ascertain the mediator role of PAF in immune complex-induced hypotension. By using the same PAF receptor antagonist in the present study, we provided evidence that PAF is also the dominant mediator in the SIg-induced hypotension and increased vascular permeability. The time delay between the SIg infusion and the onset of hypotension may reflect the time necessary for PAF production and secretion. The observation of a temporary restoration of the blood pressure after a bolus dose of L-652,731 during the hypotension indicates that the production of PAF in the rat continues during the total duration of hypotension, i.e. several hours.

Several investigators have indicated the liver and the spleen as the sites of immune complex-induced PAF production (Inarrea, Alonso & Sanchez-Crespo, 1983; Doebber, Wu & Biftu, 1986). However, because PAF may be produced by many cell types, uncertainty exists as to exactly which cell type is its major source. The macrophage depletion by the DMDP liposome treatment is radical and selective. Earlier ultrastruc-

tural studies have shown the complete disappearance of the macrophages (Van Rooijen, Van Nieuwmegen & Kamperdijk,, 1985), whereas other cell types remain. Taking into account the fact that the rats were still responsive to PAF, we conclude that PAF production by macrophages is responsible for the vascular effects. It has been documented that i.v.Ig may interfere with Fc-receptor-mediated effector functions of macrophages (Kimberly *et al.*, 1984; Jungi *et al.*, 1986). Therefore, our observation that the vascular responses are prevented by pretreatment with monomeric IgG or i.v.Ig, provides indirect evidence that the activation of the macrophages by SIg occurs via the Fc receptors.

Figure 5 summarizes schematically the hypothetical sequence of events leading to the different responses in the rat induced by SIg infusion. IgG aggregates may activate two independent effector mechanisms of the immune system—the macrophages and the complement system. The efficacy of activation of each mechanism depends on the size of aggregates. It seems clear now that the vascular responses are mediated by PAF release from macrophages. The effector mechanism of thrombocytopenia and neutropenia is, however, less clear. Probably both complement activation and PAF release lead to these phenomena.

The finding that immunoglobulin preparations may trigger *in vivo* PAF release from macrophages raises the question of whether this mechanism is also involved in the clinically observed adverse reactions. Since we studied acute events and since the hypotensive reaction was limited to certain immunoglobulin preparations, it is improbable that the reactions in rats can be attributed to infusion of a heterologous protein. As discussed previously (Bleeker *et al.*, 1987), there is some controversy in the literature concerning the mechanism of the adverse reactions to i.v. administration of immunoglobulins. Barandun & Isliker (1986) have found a correlation with complement activation in the recipient. However, adverse reactions have also been observed without significant complement depletion (Gerritz, Pirofsky & Nolte, 1976; Eibl, 1979). Therefore, Alving *et al.* (1980) postulated activation of the kinin system by prekallikrein activator as a cause of adverse reactions. These controversial clinical observations may be reconciled by assuming that IgG-aggregate-induced PAF release from macrophages is the crucial effector mechanism in the adverse effects of intravenously infused immunoglobulins. Firstly, it may explain why adverse reactions may occur without complement depletion in the patient. It has been shown by others that the IgG aggregates in immunoglobulin preparations are predominantly dimers (Tankersley, Preston & Finlayson, 1988), which are less likely to activate complement than large aggregates (Doekes, Van Es & Daha, 1982), but may still interact with macrophage Fc receptors (Huizinga *et al.*, 1989). Although SIg preparations always caused some complement depletion in the rats, we encountered several experimental batches of i.v. immunoglobulins from different manufacturers with a low anti-complementary activity *in vitro* that induced severe hypotension without significant changes in the serum haemolytic complement (unpublished observations). Secondly, it explains the observation of Barandun & Isliker (1986) that infusion of monomeric IgG to patients who have manifested adverse reactions to SIg renders them tolerant to subsequent i.v. administration of SIg. In the rat, administration of monomeric IgG or i.v.Ig had no effect on the decrease in haemolytic complement after subsequent SIg infusion; however, it completely blocked the macrophage-mediated responses. Therefore, it seems likely that inhibition of the Fc-mediated macrophage functions (Hassig, 1986) is the reason of tolerance induction in patients.

The measurement of the blood pressure reactions in pentobarbital-anaesthetized rats provides a sensitive test for macrophage-activating capacity of i.v.Ig preparations. It seems likely that macrophage-mediated PAF release is a major effector mechanism of possible side effects in patients. Therefore, the testing in rats is valuable in the safety control of i.v.Ig, in addition to *in vitro* tests such as anti-complementary activity, aggregate content and prekallikrein activator activity. The blood pressure test should be useful in the development of new i.v.Ig preparations.

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