

# Immunoglobulin M–Enriched Human Intravenous Immunoglobulin Prevents Complement Activation In Vitro and In Vivo in a Rat Model of Acute Inflammation

By Robert Rieben, Anja Roos, Yvonne Muizert, Caroline Tinguely, Arnout F. Gerritsen, and Mohamed R. Daha

An important antiinflammatory mechanism of intravenous immunoglobulin preparations (IVIg) is their ability to block complement activation. The purpose of this study was to compare the complement-inhibitory activity of four IVIg preparations differing in isotype composition. The preparations were: (1) IVIgG (48 g/L IgG, 2 g/L IgA; Intraglobin F); (2) Pentaglobin (38 g/L IgG, 6 g/L IgM, 6 g/L IgA); (3) IVIgM (35 g/L IgM, 12 g/L IgA, 3 g/L IgG); and (4) IVIgA (41 g/L IgA, 9 g/L IgG), all from Biotest Pharma GmbH, Dreieich, Germany. Their complement inhibitory activity was assessed in vitro by measurement of the blocking of C1q-, C4-, and C3 deposition on solid-phase aggregated rabbit IgG by enzyme-linked immunosorbent assay (ELISA). Complement inhibition in this ELISA was best for IVIgM, followed by Pentaglobin and IVIgG; IVIgA did not exhibit an inhibitory effect. Control experiments with excess concentrations of C1q as well as with C1q-depleted serum showed that the inhibitory effects of IVIg were not caused by complement activation

**I**NTRAVENOUS IMMUNOGLOBULIN preparations (IVIg) exert a beneficial effect in many clinical situations that are characterized or accompanied by a dysfunction or dysregulation of the immune system. Among others, these include immunodeficiencies, autoimmune diseases, sepsis, and severe trauma. Besides the effect of IgG substitution in case of a deficiency, several other mechanisms have been postulated for the beneficial effect of IVIGs: blocking of Fc receptors on proinflammatory cells,<sup>1</sup> antiidiotypic neutralization of autoantibodies,<sup>2</sup> modulation of cytokine production by lymphocytes,<sup>3,4</sup> and effects on the complement system.<sup>5</sup> The interaction of IVIG with the complement system was reported to take place at least at three different stages: Basta et al<sup>6,7</sup> reported a binding of the activated complement components C4b and C3b to the Ig molecules and Mollnes et al<sup>8</sup> found binding of C1q to the Ig molecules in an in vitro system. Both of these mechanisms divert the attack of complement from the target by scavenging active complement components. A third mechanism was recently shown by Lutz et

and thus, consumption, but that C4 and C3 were scavenged by IgM and to a lesser extent by IgG. These results were confirmed in vivo in the rat anti-Thy 1 nephritis model, in which a single dose of 500 mg/kg of IVIgM prevented C3-, C6-, and C5b-9 deposition in the rat glomeruli, whereas the effect of IVIgG was much less pronounced. Reduction of complement deposition was paralleled by a diminished albuminuria, which was completely absent in the IVIgM-treated rats. IVIgM and to a lesser extent IVIgG also prevented rat C3 deposition on cultured rat glomerular mesangial cells in vitro, but did not influence anti-Thy 1 binding. Neither IVIgM nor Pentaglobin nor IVIgG negatively affected in vitro phagocytosis of *Escherichia coli* (*E coli*) by human granulocytes. In conclusion, we have shown that IgM enrichment of IVIG preparations enhances their effect to prevent the inflammatory effects of complement activation.

© 1999 by The American Society of Hematology.

al,<sup>9</sup> who described an enhancement of the physiologic cleavage of C3b-Ig complexes. This latter effect of IVIG prevents damage by C3b-carrying immune complexes in circulation by accelerating their decay. It was recently suggested that isotype differences exist concerning the capacity of human Ig to scavenge C4b and C3b. Miletic et al<sup>10</sup> showed in vitro that human IgM and IgA have a higher capacity to bind C4b and C3b than IgG.

The aim of the present study was to compare the complement-inhibitory capacities of different IVIG preparations using in vitro and in vivo models of complement activation and to obtain further insight in the complement components involved in this process. Besides pure IgG-containing IVIG (IVIgG), we had the opportunity to include in our tests two different IVIGs, which were enriched in IgM, content as well as one IgA-enriched preparation.

## MATERIALS AND METHODS

**IVIg preparations.** Ig fractions of large pools of human plasma were obtained from Biotest Pharma GmbH (Dreieich, Germany). Four preparations were compared in the different assays: (1) IVIgG (48 g/L IgG, 2 g/L IgA; Intraglobin F); (2) Pentaglobin (38 g/L IgG, 6 g/L IgM, 6 g/L IgA); (3) IVIgM (35 g/L IgM, 12 g/L IgA, 3 g/L IgG); and (4) IVIgA (41 g/L IgA, 9 g/L IgG). The IVIG preparations were dissolved to a protein concentration of 50 g/L in a buffer containing 26 g/L of glucose, pH 6.6. Intraglobin F and Pentaglobin are commercially available, whereas IVIgM and IVIgA were laboratory preparations.

**Antibodies.** Antisera to human and rat C1q, C4, C3, and C9, respectively, were raised in goats or rabbits by repeated subcutaneous injections of the purified components and their specificity was checked by enzyme-linked immunosorbent assay (ELISA) and Western blotting. The IgG were purified from the antisera by ammonium sulfate precipitation and diethyl aminoethyl (DEAE) Sepharose column chromatography (Pharmacia Biotech, Uppsala, Sweden). Labeling of these antibodies with digoxigenin (DIG, Boehringer Mannheim GmbH, Mannheim, Germany), biotin (Pierce Chemical Co, Rockford, IL), and fluorescein isothiocyanate (FITC, Koch-Light Laboratories Ltd, Colnbrook, Bucks, UK) was performed according to the instructions of the

From the Departments of Cardiology and Hematology, Bern University Hospital, Bern, Switzerland; and the Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands.

Submitted May 8, 1998; accepted September 23, 1998.

Supported by Grant No. 823A-040153 from the Swiss National Science Foundation (to R.R.) and Biotest Pharma GmbH, Dreieich, Germany. M.R.D. and R.R. are partners in the EU Biotechnology Project on Xenotransplantation No. BIO4-CT97-2242/Swiss Federal Office for Education and Science No. 97.0369.

Address reprint requests to Robert Rieben, PhD, Department of Cardiology, University Hospital, CH-3010 Bern, Switzerland; e-mail: rieben@webshuttle.ch.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1999 by The American Society of Hematology.

0006-4971/99/9303-0031\$3.00/0

suppliers. Horseradish peroxidase (HRP)-labeled sheep F(ab')<sub>2</sub> to DIG was purchased from Boehringer Mannheim. Hybridomas secreting mouse monoclonal antibody (MoAb) specific for human IgM and IgG (clones HB57 and HB43) were obtained from the American Type Culture Collection (Rockville, MD) and the antibodies purified from cell supernatant according to standard techniques. HRP- and FITC-labeled goat anti-mouse IgG antibodies were from Southern Biotechnology Associates (SBA, Birmingham, AL). For fluorescence-activated cell sorting (FACS) analysis, the following antibodies and conjugates were used: a biotinylated mouse MoAb directed against rat C3 (C3-1), described previously,<sup>11</sup> phycoerythrin (PE)-labeled goat anti-mouse antibodies (Dako, Glostrup, Denmark), PE-conjugated streptavidin (Becton Dickinson, San Jose, CA), FITC-conjugated goat anti-human IgM (Nordic Immunologic Laboratories, Tilburg, The Netherlands), and FITC-conjugated goat anti-human IgG (De Beer Medicals, Diessen, The Netherlands).

*Analysis of the C1q-, C4-, and C3 scavenging activity in vitro.* An ELISA system was set up in which the inhibition of C1q-, C4-, and C3 deposition from normal human serum (NHS) to aggregated rabbit IgG by the different IVIG preparations was measured. Heat-aggregated rabbit IgG (aIgG, 10 mg/mL, 20 minutes at 63°C, insoluble aggregates were removed by centrifugation for 10 minutes at 3,000g) was coated to microtiter plates (NUNC Maxisorp, NUNC AB, Roskilde, Denmark) at 10 µg/mL in 0.1 mol/L carbonate buffer pH 9.6 for 2 hours at 37°C or overnight at room temperature. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20, the plates were saturated with PBS containing 0.05% Tween 20 and 2% casein (Sigma-Aldrich Co, St Louis, MO) for 1 hour at 37°C. Human serum was diluted 1:200 in half-isotonic veronal-buffered saline containing 0.05% gelatin, 0.15 mmol/L CaCl<sub>2</sub>, 0.5 mmol/L MgCl<sub>2</sub>, 0.05% Tween 20 (GVB<sup>++</sup>), mixed with a serial dilution (25 to 0.03 mg/mL) of IVIG and then put on the coated plates for 1 hour at 37°C. DIG-labeled goat antibodies to human C1q, C4, or C3, HRP-labeled sheep anti-DIG F(ab')<sub>2</sub> and ABTS substrate (Sigma) were used to reveal the deposition of C1q, C4, or C3 on the plate. The absorption at 415 nm was measured with a microplate reader.

Two different variations of this technique were used to assess whether the inhibitions of C4- and C3-binding were independent of the inhibition of C1q-binding to the aIgG coat: (1) the human serum was diluted in C4-deficient guinea pig serum (end-concentration in the assay: NHS 1:200, guinea pig serum 1:2) instead of GVB<sup>++</sup>, ensuring an excess of all complement components besides C4. (2) Purified human C1q (produced at our own laboratory as described earlier)<sup>12</sup> was added to the aIgG coated plates at a concentration of 7 µg/mL in GVB<sup>++</sup>, the plates washed, and subsequently 1:200 diluted C1q-depleted NHS used for the assay. The C1q depletion of this serum was performed by absorption on an IgG-Sepharose column and the levels of all complement components besides C1q were in the normal range, as assessed by hemolytic assays. Bound C1q, C4, and C3 to the aIgG/C1q-coated plate was detected as described above.

*Assessment of in vitro C3a generation by IVIgG and IVIgM in human serum.* Aliquots of fresh human serum were incubated with serial dilutions of either IVIgG, Pentaglobin, or IVIgM (100 µL serum + 100 µL of IVIG diluted in veronal-buffered saline containing 0.15 mmol/L CaCl<sub>2</sub> and 0.5 mmol/L MgCl<sub>2</sub> (VBS<sup>++</sup>) from 25 to 0.03 mg/mL) for 1 hour at 37°C. After the incubation, the C3a contents of the samples were immediately quantitated using an ELISA (Quidel, San Diego, CA). As controls, serum was also incubated with heat aggregated IVIgG (20 minutes, 63°C) and VBS<sup>++</sup> alone.

*Binding of C4 to human Ig.* After preincubation of the IVIG with human serum and incubation on the plate coated with aIgG, binding of C4 to human IgM and IgG was analyzed by sandwich ELISA. Microtiter plates (NUNC Maxisorp) were coated with goat anti-human C4 at 2 µg/mL in 0.1 mol/L carbonate buffer pH 9.6. After saturation and washing of the plates, the supernatant of the above-described test

(hemolytically active human serum 1:200 with a serial dilution of IVIG) was incubated for 1 hour at 37°C. After washing, human IgM and IgG, which were bound to the plate were revealed isotype-specifically by MoAb and goat anti-mouse HRP conjugate.

*IVIG in the rat anti-Thy 1 nephritis model.* The induction of acute glomerular nephritis in the rat by IV injection of mouse IgG2a MoAb (ER4G) against the rat Thy 1.1 antigen has been described earlier.<sup>13</sup> We used this model to investigate the potential of IVIG to prevent complement activation and inflammation in vivo. Inbred male Wistar rats, weighing approximately 200 g, were divided into three groups of four rats each. All rats received an IV injection of 2 mL of either PBS (group 1, control group), IVIgG (500 mg/kg, group 2), or IVIgM (500 mg/kg, group 3); all IV injections were given in the tail vein. Thirty minutes after the injection with PBS or IVIG, the rats were administered IV 0.5 mg/kg of ER4G in 0.5 mL PBS. Kidney biopsies were taken after 10 minutes, 30 minutes, and 24 hours, and the animals were killed after the last biopsy. All tissue samples were snap-frozen in precooled isobutanol and stored in liquid nitrogen until analyzed further. Blood samples were taken before the injection of IVIG (time = -30 minutes), before injection of ER4G (t = 0 min), and at the same time as the biopsies (t = 10 minutes, 30 minutes, and 24 hours). The blood samples were kept on ice during the experiment, centrifuged, and the sera stored at -80°C. The sera were used later to immunochemically determine the levels of rat C1q, C3, C6, and C5b-9, as well as human IgM and IgG.

For the assessment of albuminuria, another three groups of three Wistar rats each were injected according to the same scheme as above, but no biopsies were performed. In addition, one rat each was injected with 500 mg/kg IVIgG and IVIgM at t = -30 minutes followed by 0.5 mL of PBS instead of ER4G to detect any albuminuria caused by IVIG. Urine was collected from 0 to 4 hours and 4 to 24 hours after injection of the anti-Thy 1 MoAb with ≈1% merthiolate (Koch-Light Laboratories) and stored frozen at -20°C until the albumin contents were measured.

*Detection of deposition of complement components, as well as human Ig in kidney tissue.* Snap-frozen renal tissue was cut into 2-µm sections, air dried, and acetone fixed for 10 minutes at 22°C. Slides were washed twice for 7 minutes in PBS and immunofluorescence was performed either directly using FITC-conjugated antibodies or DIG-conjugates followed by HRP-labeled sheep anti-DIG F(ab')<sub>2</sub> (Boehringer Mannheim) and tyramide-FITC conjugate (NEN Life Science, DuPont de Nemours B.V., Dordrecht, The Netherlands). The following antibodies were used: (1) detection of bound anti-Thy 1 MoAb: goat anti-mouse IgG2a-FITC (Nordic, Tilburg, The Netherlands); (2) detection of rat C1q: rabbit F(ab')<sub>2</sub> anti-rat C1q (produced at our own laboratory) followed by HRP-conjugated goat anti-rabbit IgG (Boehringer Mannheim); (3) detection of rat C3: rabbit anti-rat C3-FITC (produced at our own laboratory); (4) detection of rat C6: DIG-labeled mouse MoAb specific for rat C6 (produced at our own laboratory); and (5) detection of rat C5b-9: DIG-labeled mouse MoAb specific for a rat C5b-9 neoantigen (courtesy of W.G. Couser, see Schulze et al<sup>14</sup>).

Deposition of human IgG was visualized by DIG-labeled HB43 MoAb, followed by FITC-labeled sheep anti-DIG F(ab')<sub>2</sub> (Boehringer Mannheim), and human IgM was detected by biotinylated HB57 MoAb and streptavidin-FITC conjugate (Amersham).

*Measurement of complement components in rat serum.* Rat C4- and C3 concentrations were determined by rocket-electrophoresis using monospecific antisera as described earlier.<sup>15</sup> In brief, rabbit anti-rat C4 and C3, respectively, were diluted 1:30 in 1% agarose solution. Subsequently, the agarose gels were poured onto glass plates and allowed to set. Wells were punched in the agarose and filled with 1:4 diluted samples and a serial dilution of pooled normal rat serum as a standard. The samples were subjected to electrophoresis for 4 hours at 30 mA and the plates then rinsed in PBS containing 2 mmol/L EDTA. Precipitation arcs were stained with amido black (Sigma) and the C4 and C3 concentrations of the samples calculated by comparison of their rocket heights with the ones of the standards.

**Measurement of albuminuria in rat urine.** Proteinuria was assessed by quantitation of rat albumin in a rocket electrophoresis. Rabbit anti-rat albumin antiserum for the rocket electrophoresis (produced at our own lab) was used at a dilution of 1:100 and, otherwise, the test was performed as described above for the rat C4 and C3 determination.

**Quantitation of human IgM and IgG in rat serum.** The concentrations of human IgM and IgG were measured in the serum samples of the rats treated with IVIgM. ELISA plates (NUNC maxisorp) were coated with either rabbit anti-human IgM or IgG (DAKO A/S, Glostrup, Denmark). After washing and saturation of the plates, the sera were incubated at a 1:16,000 dilution for 1 hour at 37°C. Biotinylated mouse MoAbs against human IgM (HB 57) and IgG (HB 43), followed by streptavidin-HRP conjugate (Amersham) and ABTS substrate, were used to show bound human IgM and IgG antibodies. A human serum with known concentrations of IgM and IgG was included in the test as a standard.

**Binding of anti-Thy 1 MoAb and rat C3 to cultured rat glomerular mesangial cells.** Mesangial cells (RMC) were isolated from glomeruli of Sprague Dawley rats and cultured in vitro as described.<sup>16</sup> IVIgM and IVIgG were serially diluted in culture medium (RPMI containing penicillin, streptomycin, and 10% fetal calf serum [FCS]) from 10 to 0.6 mg/mL and added to RMC grown in 24-well plates to subconfluence. After a 30-minute incubation at 37°C, anti-Thy 1 MoAb ER4G was added at a final concentration of 1 µg/mL, representing a nonsaturating concentration of ER4G. After another incubation for 30 minutes, rat serum (Wistar) was added to a final concentration of 10%, followed by incubation for 60 minutes. These conditions were chosen to minimize cell lysis during the incubation period. The cells were then harvested using 20 mmol/L EDTA in PBS and binding of ER4G and C3, as well as human IgM and IgG, were analyzed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ). Five minutes before measurement, propidium iodide (PI) was added at 1 µg/mL, and only cells being impermeable for PI were gated for analysis.

**Granulocyte phagocytosis assay.** The phagocytosis of *E coli K12* bacteria by human granulocytes was investigated using the commercially available Phagotest from OrpegenPharma (Heidelberg, Germany). The test was performed as described previously,<sup>17</sup> using blood from healthy volunteer donors and nonopsonized, FITC-labeled *E coli*. Heparinized whole blood was centrifuged to separate plasma from cells, and the latter were subsequently washed three times with PBS pH 7.4. IVIG preparations were mixed with an equal volume of the fresh plasma, resulting in an IVIG concentration of 25 mg/mL. After addition of the washed, unfractionated blood cells and the labeled *E coli* bacteria, the reaction mixture was incubated for 10 minutes at 37°C. The ratio of *E coli* to white blood cells was 25:1. Internalization of *E coli* by granulocytes was visualized by FACS (Becton Dickinson) and analysis of these measurements performed with the CELL Quest software version 3.0.1 (Becton Dickinson). Phagocytosis activity was expressed as the percentage of granulocytes that had internalized FITC-labeled *E coli*.

## RESULTS

**Prevention of complement deposition on solid-phase aggregated rabbit IgG in vitro.** In the ELISA system with hemolytically active human serum, a dose-dependent inhibition of deposition of the complement components C1q, C4, and C3 on aIgG could be observed for IVIgM, Pentaglobin, and IVIgG. IVIgM was most effective in preventing complement deposition, followed by Pentaglobin and IVIgG, whereas the IVIgA preparation did not show an inhibitory effect (Table 1). Dose response curves of the inhibition experiments with IVIgM and IVIgG are given in Fig 1. About the same concentrations of IVIG were needed for 50% inhibition of C4 and C3 binding

**Table 1. Inhibition of Complement Deposition on Aggregated Rabbit IgG by Different IVIG Preparations**

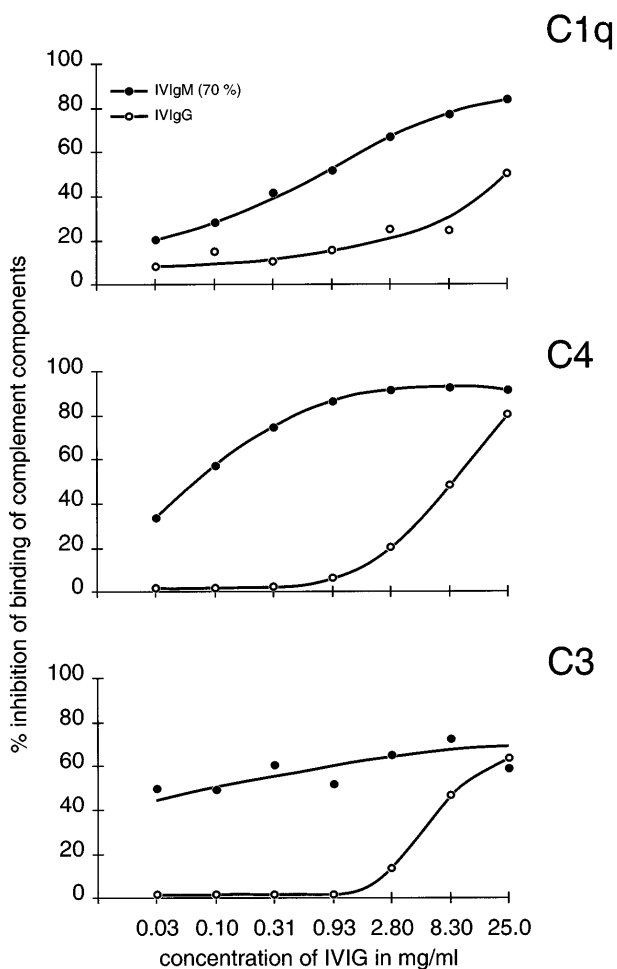
	Complement Component		
	C1q	C4	C3
IVIgM			
25 mg/mL	84*	92	70
1 mg/mL	52	87	53
Pentaglobin			
25 mg/mL	83	93	74
1 mg/mL	42	79	64
IVIgG			
25 mg/mL	52	86	56
1 mg/mL	15	4	1
IVIgA			
25 mg/mL	0	0	0
1 mg/mL	10	0	0

Human serum, diluted 1:200 in GVB<sup>++</sup>, was incubated on a plate coated with rabbit aIgG together with a serial dilution of the different IVIG preparations. After the incubation, deposition of C1q, C4, and C3 on the aIgG were measured by ELISA. The values given are the percentages of inhibition of complement deposition that were reached with 1 and 25 mg/mL of IVIG, respectively.

\*Percent inhibition.

(Table 2), but with the difference that binding of C4 could be blocked to greater than 90% with the IgM-containing preparations (IVIgM and Pentaglobin), whereas C3 binding could only be blocked to a maximum of 75%. Higher concentrations of IVIG were needed to block C1q binding in the ELISA system and the difference in inhibitory capacity between IgM-containing preparations and IVIgG was not as pronounced in this case as for blocking C4 and C3 binding.

Two sets of experiments were performed to check whether complement activation by the IVIG preparations was involved in the observed inhibitions of C1q, C4, and C3 binding to aIgG: first, C4-deficient guinea pig serum was used for the 1:200 dilution of the human serum to have an excess of all complement components available besides C4, the binding of which was measured on the aIgG-coated plate. In this experiment, the inhibition curves for C4-binding were similar to the ones observed without guinea pig serum (Fig 1), but higher amounts of IVIG were needed to obtain 50% inhibition (Table 2). Second, another modification of the inhibition ELISA was used in which purified human C1q was added to the aIgG-coated plate and C1q-depleted, 1:200 diluted human serum used as the complement source. Also, in this system, a dose-dependent inhibition of C4 and C3 binding was observed, which was best for IVIgM and Pentaglobin, followed by IVIgG and IVIgA (Fig 2). In contrast to the experiment with whole, hemolytically active human serum, the inhibitory capacities of IVIgM and Pentaglobin were almost equal and also IVIgA showed some inhibitory effect. In addition, the in vitro complement activation by IVIgG, Pentaglobin, and IVIgM was assessed by quantitating C3a concentrations after a 30-minute incubation of the IVIGs with human serum. As shown in Fig 3, all tested IVIG preparations were equally active to induce C3a in human serum. Only at the highest concentration of IVIG used in this test (12.5 mg/mL), the C3a levels were significantly elevated ( $P < .01$ , Student's *t*-test) as compared with serum



**Fig 1.** Inhibition of complement deposition on solid-phase bound rabbit algG by IVIG I. Dose response curves of the effect of IVIgM and IVIgG on in vitro complement deposition. Human serum diluted 1:200 in GVB<sup>++</sup> was incubated for 1 hour at 37°C in algG-coated wells together with a serial dilution of IVIgM or IVIgG. Solid-phase bound C1q, C4, and C3 were then detected with specific DIG-labeled goat antibodies, sheep F(ab')<sub>2</sub> anti-DIG HRP conjugate and ABTS substrate. A representative experiment of three with similar results is shown.

incubated for 30 minutes with VBS<sup>++</sup> buffer alone. Heat-aggregated IVIgG, used as a complement-activating control, already led to elevated C3a levels at a concentration of 0.15 mg/mL.

**Binding of C4 to human IgM and IgG.** After incubation on the algG-coated plates, the supernatants of an inhibition test (human serum diluted 1:200 plus a serial dilution of IVIG) were analyzed for binding of C4 to human IgM and IgG. For IVIgG, a binding of C4 to IgG was observed, which mirrored the decrease of C4 deposited on the algG-coated plate. The higher inhibitory capacities of the IgM-containing IVIG preparations Pentaglobin and IVIgM on C4-deposition to algG were associated with binding of C4 to IgM, which was most pronounced in the case of IVIgM. For IVIgA, which did not measurably inhibit the deposition of C4 on algG, some binding of C4 to IgG was observed, which is its main constituent besides IgA. A column

**Table 2. Comparison of the Inhibitory Capacity (ID 50) of the Different IVIG Preparations on Complement Deposition in an In Vitro Assay**

	IVIG Preparation			
	IVIgM	Pentaglobin	IVIgG	IVIgA
NHS				
C1q	0.80	1.70	25	—*
C4	0.08	0.30	8	—
C3	0.08	0.40	11	—
NHS + C4-def. gp†				
C4	0.30	1.80	16	—
C1q-depleted NHS				
C4	0.90	0.60	9	19
C3	0.40	0.60	7	16

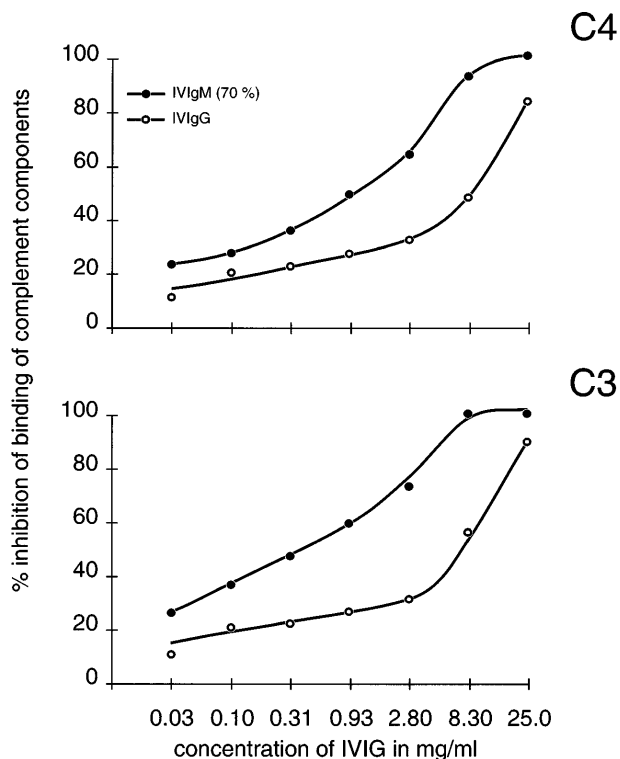
The amount of IVIG in mg/mL is given, which was necessary to obtain a 50% inhibition (ID 50) of the binding of the respective complement component to the coated algG.

\*No inhibition.

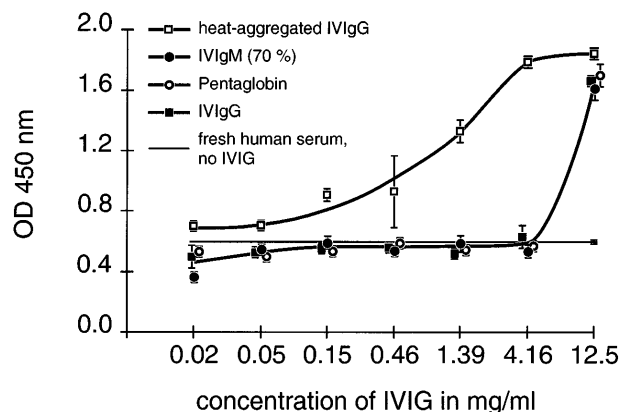
†C4-deficient guinea pig serum.

graph representation of the results obtained with an IVIG concentration of 2.8 mg/mL is given in Fig 4.

**Inhibition of complement deposition in the rat anti-Thy 1 nephritis model.** IVIgG and IVIgM were injected IV at a concentration of 500 mg/kg into rats 30 minutes before injection of 0.5 mg/kg anti-Thy 1.1 MoAb ER4G. Whereas in



**Fig 2.** Inhibition of complement deposition on solid-phase bound rabbit algG by IVIG II. After coating of the microtiter plates with algG, purified C1q was added as a second step, the plates washed, and the inhibition experiments with IVIgM and IVIgG as shown in Fig 1 for C4 and C3 deposition were then performed in 1:200 diluted, C1q-depleted human serum. A representative experiment of three with similar results is shown.



**Fig 3.** C3a generation in human serum by IVIG. A total of 100  $\mu$ L of serial dilutions of the IVIG preparations in VBS<sup>++</sup> was incubated for 1 hour at 37°C with 100  $\mu$ L of fresh human serum and the C3a contents of this incubation mixture immediately quantitated by a commercial ELISA. As controls, the baseline C3a content of the used serum and the C3a generation by heat-aggregated IVIgG are also depicted in the figure. Average values of duplicates are given with indication of the standard deviations (SD).

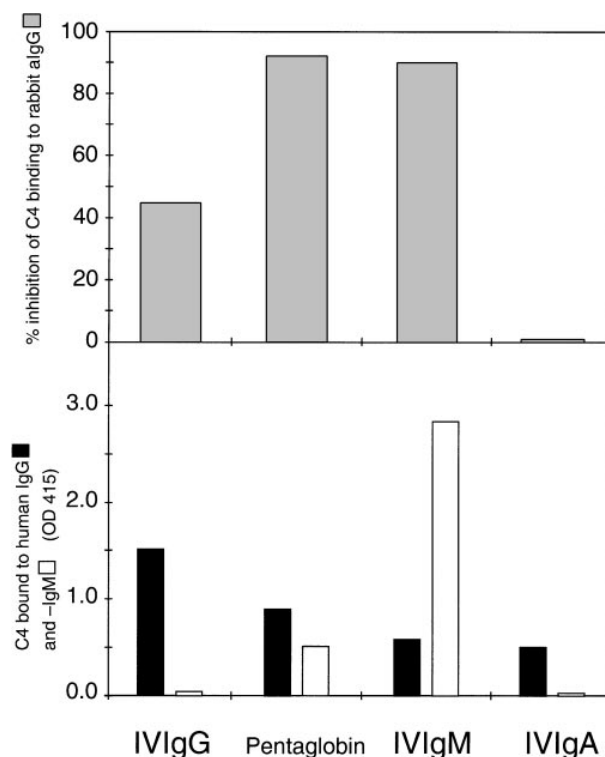
the rats that received PBS instead of IVIG deposition of complement components (C1q, C3, C6, and C5b-9) in the glomeruli could be observed by immunofluorescence as early as 10 minutes after the injection of the ER4G MoAb, this deposition was virtually absent in rats that received IVIgM (Fig 5, Table 3). A diminished complement deposition, as compared with the control group receiving PBS instead of IVIG, was observed in the case of IVIgG-treated rats. In the control group, the complement deposition was visible in all biopsy samples, ie, after 10 minutes, 30 minutes, and 24 hours. The protection from complement deposition by the IVIG preparations did not change with time during the 24-hour observation period; IVIgM almost completely prevented complement deposition as assessed by immunofluorescence, and IVIgG treatment led to a staining intermediate between the one seen in the PBS control and the IVIgM-treated rats (Table 3). No change of fluorescence intensity for ER4G MoAb binding by IVIG could be observed (Fig 5, top row).

In the IVIgG-treated group, deposition of human IgG could be observed on the glomeruli, as well as in the interstitial area on all biopsy samples. Staining for both human IgG and IgM was weakly positive on biopsy specimens of the IVIgM-treated rats (Table 3).

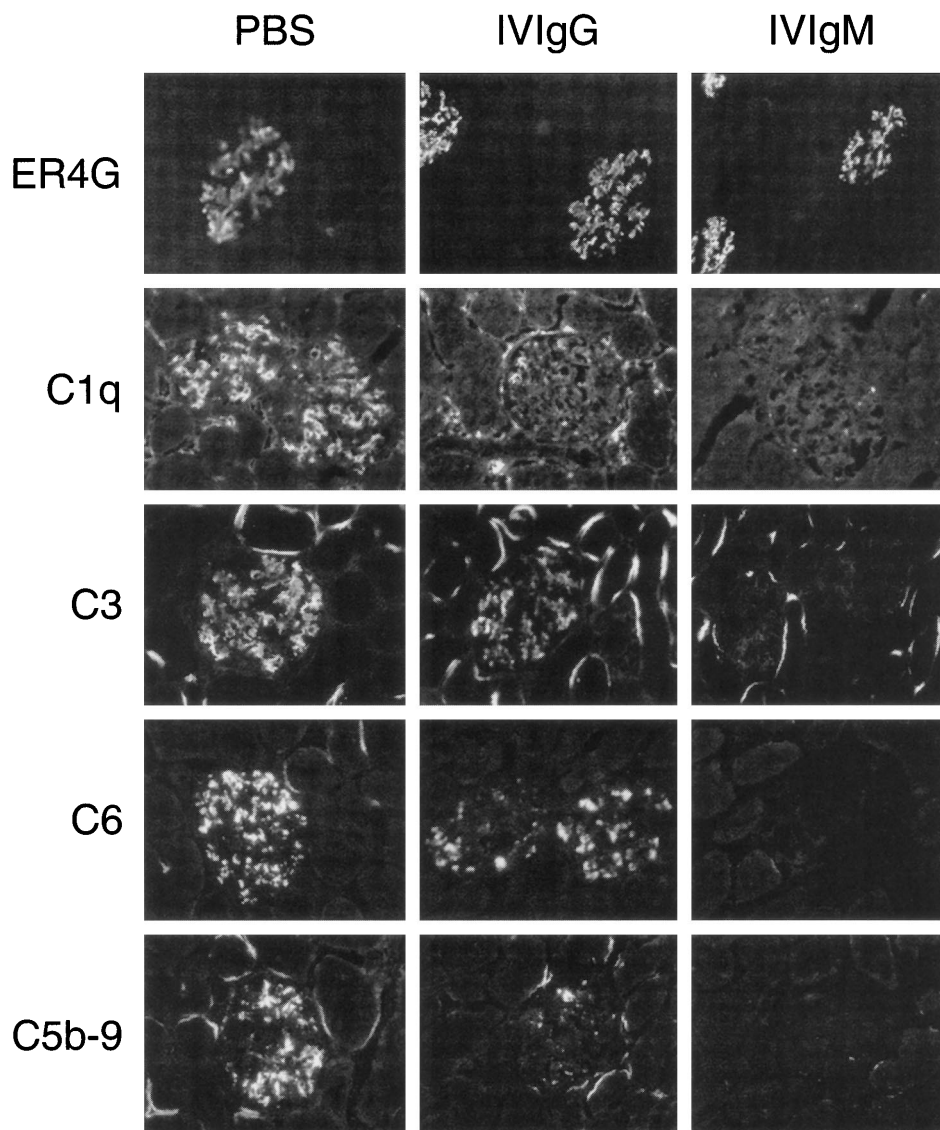
**Influence of IVIG treatment on anti-Thy 1-induced proteinuria.** Albuminuria was measured in groups of three rats each receiving either PBS, IVIgG, or IVIgM 30 minutes before injection of ER4G. Significant albuminuria was found in the 4- to 24-hour samples, and the mean total albumin contents are depicted in Fig 6. We found that injection of IVIgM prevented the anti-Thy 1-induced albuminuria and that IVIgG treatment led to an approximately two thirds reduction as compared with the control group receiving PBS. Control rats that were treated with IVIG only, without injection of anti-Thy 1 MoAb, showed albuminuria of 80 to 140  $\mu$ g during the 4- to 24-hour period after the injection, which is not significantly different from healthy Wistar rats (results not shown).

**C4 and C3 levels in sera of rats treated with IVIG and anti-Thy 1 MoAb.** To assess whether complement consumption was involved in the effects of IVIG in the anti-Thy 1 model, the serum levels of rat C4 and C3 were quantitated. Serum levels of C4 decreased to approximately 50% at  $t = 30$  minutes in the PBS control group ( $P < .05$  by  $t$ -test), whereas no significant change of circulating C4 was found in the IVIgG and IVIgM groups (Fig 7). For C3, a similar picture was seen, but with a less pronounced decrease of C3 in the PBS control group as compared with C4 (results not shown).

**Concentrations of human IgM and IgG in rat sera after injection of IVIgM and IVIgG.** The circulating levels of human IgM and IgG in the sera of the rats that received 500 mg/kg of either IVIgM or IVIgG were measured by sandwich ELISA. At the time of anti-Thy 1 injection (ie, 30 minutes after IVIgM infusion), human IgM concentrations were at 8 mg/mL and then decreased to  $\approx 5$  mg/mL within another 30 minutes and reached 2 mg/mL after 24 hours. Serum levels of human IgG were at 2 mg/mL 30 minutes after IVIgG infusion and decreased linearly to  $\approx 1$  mg/mL within 24 hours, resulting in approximate circulation half-lives of 12 hours for IgM and 24 hours for IgG (results not shown).



**Fig 4.** Binding of C4 to human IgG and IgM in the IVIG preparations. Inhibition experiments for C4 binding to coated algG were performed as described for Fig 1. Percents of inhibition of C4 binding to the algG coat at a dose of 2.8 mg/mL IVIG are given in the top panel (■, representative experiment of three with similar results). The supernatants of the incubation mixtures were then removed and incubated on a plate coated with goat anti-C4 antibody. Human IgG (■) and IgM (□) bound to this anti-C4 coat were then shown by MoAb and goat anti-mouse HRP conjugate. The OD 415 values (averages of duplicates) of the latter assay are given in the bottom panel.



**Fig 5.** Deposition of ER4G, rat C1q, C3, C6, and C5b-9 in kidney biopsy specimens. Groups of four rats each were injected IV 500 mg/kg of IVIG and 30 minutes later 0.5 mg/kg anti-Thy 1 MoAb, ER4G. Kidney biopsies were performed 10 minutes, 30 minutes, and 24 hours after ER4G injection and analyzed by immunofluorescence for the presence of ER4G and rat complement components. The extraglomerular staining observed for C3 is frequently encountered in kidney sections of adult rats and not related to the anti-Thy 1 injection. Magnification was 250:1 for all micrographs.

*Influence of IVIG on binding of anti-Thy 1 MoAb and rat C3 to cultured rat mesangial cells.* To check the possibility that IVIG might compete with the ER4G MoAb for binding sites on glomerular mesangial cells, we used an in vitro assay with cultured RMC. The binding of ER4G, as well as human IgM and IgG on RMC, was quantitated by FACS analysis. ER4G bound to RMC at a high level, showing mean fluorescence intensities (MFI) >1,500. Furthermore, a dose-dependent binding of the human Igs could be detected. As shown on the upper panel of Fig 8, preincubation of RMC with IVIG did not inhibit binding of ER4G. In the same experiment, binding of rat C3 to RMC was also measured. Similar to the experiments with aggregated IgG, a dose-dependent inhibition of C3 binding to RMC was observed, which was more pronounced for IVIgM than for IVIgG (Fig 8, lower panel).

*Effect of IVIG on in vitro phagocytosis of E coli by human granulocytes.* A commercially available phagocytosis test was used to assess the phagocytic function of human granulocytes at a high concentration of IVIG. Compared with the effect

of heat-inactivation of the plasma, only a minimal inhibitory effect on phagocytosis could be observed with the tested IVIG preparations. The results, expressed as the percentage of granulocytes that had ingested FITC-labeled *E coli* bacteria, are represented in Table 4.

#### DISCUSSION

The results presented in this study show that IgM enrichment of IVIG preparations leads to an enhanced complement-inhibitory capacity both in vitro and in vivo as compared with pure IVIgG. We developed an ELISA in which the inhibitory effect of IVIG on deposition of the components involved in the classical pathway of complement activation could be quantitated. The 70% pure IVIgM preparation was clearly the best inhibitor of C4 and C3 deposition in this ELISA system, followed by Pentaglobin, which contains 12% IgM, and standard,  $\geq 95\%$  pure IVIgG (Intraglobin). In contrast to earlier reports by others,<sup>10</sup> we could not find an enhanced complement-inhibitory activity of IVIgA as compared with IVIgG.

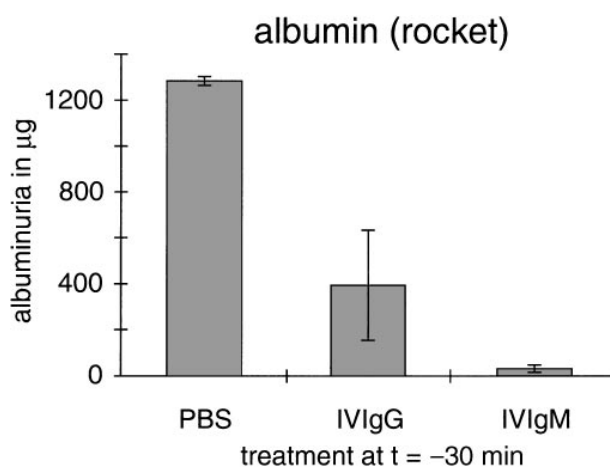
**Table 3. Detection of ER4G, Rat Complement Components, and Human Ig on Kidney Biopsy Samples by Immunofluorescence Staining**

	ER4G	C3	C5b-9	hIgG	hIgM
PBS*	+++	++	+	-	-
IVIgG	++	±	±	+	-
IVIgM	++	-	-	+ ± -	+ ± -

\*Treatment 30 minutes before injection of ER4G.

†Intensity of immunofluorescence staining scored as -, no stain; ±, <1/4; +, 1/4 to 1/2; and ++, >1/2 of glomerulus stained on the average; 20 glomeruli were scored per biopsy. Results are given per group of four rats and indicated separately for 10-minute, 30-minute, 24-hour biopsies where time-dependent differences were observed; interindividual differences were always less than one scoring unit. Groups of four rats each were injected with either PBS, IVIgG, or IVIgM followed by anti-Thy 1 MoAb, ER4G. Biopsy samples of kidneys were taken 10 minutes, 30 minutes, and 24 hours thereafter and stained for the presence of ER4G, rat complement, and human Igs as described in Materials and Methods.

The scavenging of C4b and C3b was reported by some investigators to be independent of C1q, the recognition phase of the classical complement cascade,<sup>18</sup> whereas others challenged this finding by showing that the complement-inhibitory capacity of IVIG was mainly dependent on competitive binding of C1q to Ig molecules and only to a lesser extent on C4 and C3 binding.<sup>8</sup> In our ELISA system, we found both inhibition of C1q and C4/C3 binding, and we therefore designed three experiments to see whether binding of C1q to the Ig molecules in the IVIG preparations led to complement activation and, thus, consumption: (1) A 100-fold excess of C1q over C4 as compared with the relations in serum was used by diluting the human serum in C4-deficient guinea pig serum. (2) By adding purified C1q to the coated aIgG and then using C1q-depleted human serum as the source of C4 and C3, the effects of scavenging and complement activation were locally separated.



**Fig 6. Anti-Thy 1-induced albuminuria of rats treated with PBS (control), IVIgG, or IVIgM.** Groups of three rats each were injected 500 mg/kg of IVIgG, IVIgM, or 2 mL of PBS, followed after 30 minutes by 0.5 mg/kg ER4G. Urine was collected from 4 to 24 hours after ER4G injection and the albumin content quantitated by rocket electrophoresis. Single determinations for each of the rats were performed, mean values per group and SD are shown. Differences between groups were statistically significant ( $P < .05$ ) by Student's *t*-test.

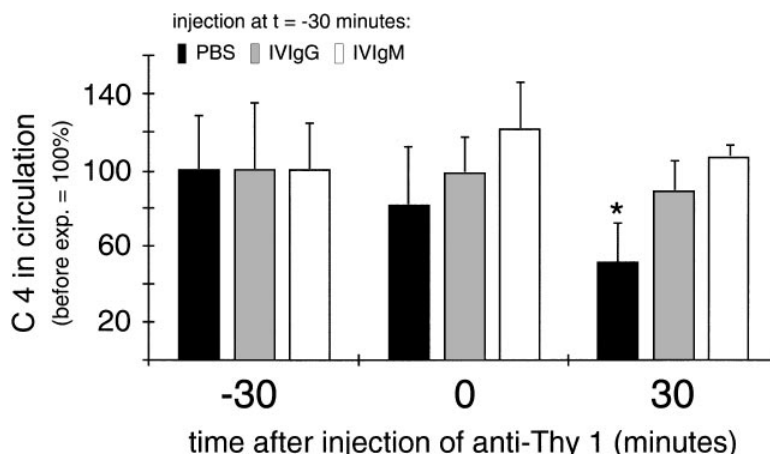
(3) The C3a generation by IVIG in human serum was quantitated by an ELISA.

For experiment (1), the resulting concentrations necessary for 50% inhibition (ID 50) of C4-binding were only twofold to sixfold higher than with 1:200 diluted human serum alone. As in this system, a 100-fold excess of C1q was present as compared with the original experiment, a strict dependency of the inhibition of C4-binding to aIgG on C1q-dependent complement activation by IVIG seems unlikely. Experiment (2) was designed in such a way that the absence of C1q in solution only allowed complement activation on the solid phase, whereas scavenging of C4 and C3 is a fluid phase event. As compared with the experiment with whole, hemolytically active serum, the ID 50 was  $\approx 10$  times higher for IVIgM, whereas the values were not significantly elevated for Pentaglobin and IVIgG. Interestingly, IVIgA showed inhibition of complement deposition under these conditions, although its action was the weakest of all tested IVIGs. Finally, with experiment (3) we showed that generation of C3a on incubation of IVIG with normal human serum was equal for IVIgG and the IgM-containing preparations Pentaglobin and IVIgM. For all of the preparations we could only find a significant generation of C3a at a concentration of 12.5 mg/mL, indicating that the *in vitro* complement-activating capacity of the IgM-containing preparations is not higher than the one of IVIgG.

That binding of complement components to the Ig molecules, scavenging, was the mechanism by which IVIG prevented their deposition on the activating surface was directly shown for C4. All inhibitory active IVIG preparations (IVIgM, Pentaglobin, and IVIgG) showed binding of C4 to IgM and/or IgG after incubation with serum in the aIgG-coated microtiter plate. The stronger inhibitory effect of the IgM-containing preparations was associated with a higher amount of C4 bound to IgM as compared with IgG (Fig 4). Taken together, the *in vitro* experiments showed that (1) IVIgM and IVIgG were able to block deposition of C1q, C4, and C3 on a surface activating the classical complement cascade, (2) a higher concentration of IgM in the preparation enhanced this effect, (3) scavenging of complement components rather than complement activation and consumption was the underlying mechanism of the inhibitory effect.

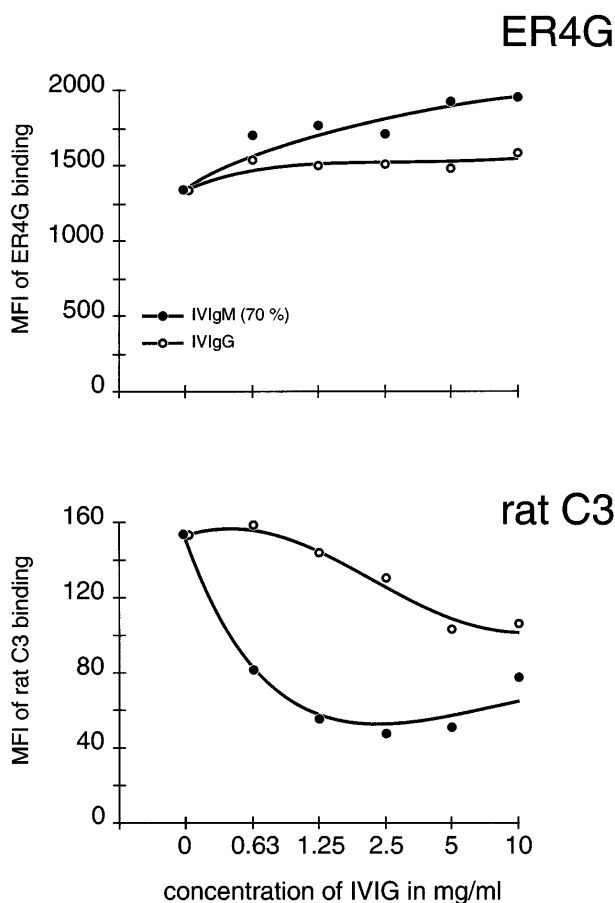
The anti-Thy 1-induced nephritis model in the rat was chosen to validate our *in vitro* data in an *in vivo* system. In this model, binding of the anti-Thy 1 MoAb, ER4G, to its respective antigen on glomerular mesangial cells leads to complement activation via the classical pathway and subsequently inflammation, which is associated with proteinuria. At an ER4G dose of 1 mg/kg, this induced proteinuria is transient, reaching maximum levels at day 3 after injection and then slowly regressing to normal levels after 3 weeks.<sup>19</sup> The ER4G concentration of 0.5 mg/kg, which we used in our study, was chosen to result in a clear-cut complement deposition, as well as albuminuria, but to be able to detect and compare beneficial effects of IVIGs without having an excess of inflammatory stimulus available. A comparison of IVIgG and IVIgM in this system showed a much better complement inhibitory and antiinflammatory effect of the latter. Both complement deposition and albuminuria were almost completely prevented by IVIgM, whereas only partial protection from these effects was provided by IVIgG.

**Fig 7.** Circulating C4 levels in rats after IVIG treatment and subsequent injection of anti-Thy 1 MoAb. Sera of the rats used in the experiment described in Fig 5 were used for quantitation of circulating complement levels. The concentration of C4 was quantitated by rocket electrophoresis using a monospecific rabbit anti-rat C4 antiserum. The asterisk (\*) indicates a significant reduction of C4 as compared with t = -30 minutes (Student's *t*-test, *P* < .05). Columns are mean values of duplicate measurements with indication of the SD.



Quantitation of C4 and C3 levels in the rat sera showed that neither PBS nor IVIgG- or IVIgM injection by itself led to a consumption of C4 or C3 (Fig 7, t = 0 minutes). However, after injection of ER4G, the expected consumption of these compo-

nents (especially of C4) could be observed in the control group receiving PBS, whereas the rats treated with IVIgG or IVIgM were protected from ER4G-induced complement consumption. These data are in accordance with the results on complement deposition on the biopsies, showing a reduction of C4 and C3 deposition in the IVIgG, and notably the IVIgM, but not the PBS group.



**Fig 8.** Influence of IVIG on anti-Thy 1 binding and rat C3 deposition on rat glomerular mesangial cells (RMC) in vitro. Cultured RMC were incubated with serial dilutions of IVIgM and IVIgG followed by addition of anti-Thy 1 MoAb, ER4G (1  $\mu$ g/mL) and 10% normal rat serum. Binding of ER4G (top) and rat C3 (bottom) was analyzed in parallel by FACS. A representative experiment of two performed is shown.

To determine whether IVIG might compete with ER4G for Thy 1 antigen on the glomerular mesangial cells, we performed in vitro experiments with cultured rat mesangial cells. Neither IVIgM nor IVIgG influenced the binding of the ER4G MoAb to RMC as assessed by FACS analysis, whereas a dose-dependent inhibition of rat C3 binding was also observed in this in vitro model. As in the experiments with aggregated IgG, the inhibition of C3 binding was better for IVIgM as compared with IVIgG. As in the rat experiments, the dose of ER4G (1  $\mu$ g/mL) that was used in this assay was titrated to be nonsaturating and the highest IVIG concentration (10 mg/mL) was equal to or higher than the maximum serum levels for human Ig that were achieved in vivo (8 mg/mL for human IgM and 2 mg/mL for IgG). Besides proving in vitro that binding of ER4G to RMC was not affected by IVIG, these results also indicate that human IVIG has a similar effect on rat complement activation in vitro as it has on the activation of human complement. A potent inhibition of rat complement activation by the different human

**Table 4.** Effect of Different IVIG Preparations on In Vitro Phagocytic Activity of Human Granulocytes

Test Sample	Phagocytosis-Efficiency of Granulocytes
Plasma + IVIgM*	72.3 $\pm$ 0.81
Plasma + Pentaglobin	73.2 $\pm$ 0.8
Plasma + IVIgG	73.6 $\pm$ 0.7
Plasma alone	78.0 $\pm$ 0.6
Heat-inactivated plasma	17.5 $\pm$ 0.5
PBS	1.2 $\pm$ 0.4

\*Concentration of IVIGs: 25 mg/mL.

†Expressed as percentage of granulocytes that had ingested FITC-labeled *E coli*, with indication of the standard deviation. IVIG preparations were mixed with an equal volume of fresh plasma, added to unfractionated blood cells and FITC-labeled *E coli* and incubated for 10 minutes at 37°C. Phagocytosis activity of granulocytes was analyzed by FACS.



IVIG preparations, best again for IVIgM, was also observed in an assay for total complement activity (Autokit CH50, Wako Pure Chemical Industries, Osaka, Japan; results not shown).

One important reason for the clinical use of IVIG today is its antibacterial activity. The observation of IVIG-mediated complement inhibition, therefore, prompted investigations into the influence of IVIG on complement-bacteria interactions. It was recently shown by Wagner et al<sup>20</sup> that IVIgG did not inhibit complement deposition on a number of different bacterial strains. Granulocyte phagocytosis is a major physiologic consequence of bacterial opsonization by Ig and complement; therefore, we decided to measure the impact of our IVIG preparations on in vitro phagocytosis of *E coli K12* by human granulocytes. In contrast to heat inactivation of the used plasma, addition of IVIG at 25 mg/mL had only a minimal effect on phagocytosis and no difference between IVIgG and the IgM-enriched preparations could be observed (Table 4). Although more experiments in this direction with different types of bacteria and phagocytes of different healthy donors and patients will have to be performed, we judge these results as preliminary evidence that IgM enrichment of IVIG will not negatively influence phagocytosis of bacteria by human granulocytes.

Today, in fact, IgM enrichment of IVIG is mainly looked at as an improvement of its antibacterial activity, primarily because of the opsonizing effect of IgM.<sup>21</sup> Clinical studies with bone marrow transplant recipients<sup>22,23</sup> and also experience with intensive care patients<sup>24-27</sup> have proven the safety and efficacy of the IgM-enriched preparation Pentaglobin. In view of our results and the ones of others,<sup>10, 28</sup> we can think of an extension of the indications of IgM-enriched IVIG, especially of preparations with a high concentration ( $\geq 50\%$ ) of IgM. The use of such an IVIgM might be beneficial in many clinical situations in which the blocking of complement activation is crucial, ranging from autoimmunity to vascular allograft or xenograft rejection. One could speculate that in the future IgM-enriched preparations will be used as a complementation of other antiinflammatory treatments.

#### ACKNOWLEDGMENT

We thank Daniëlle van Gijlswijk-Jansen and Ria Faber-Krol (Leiden) and Katja Dzelalija (Bern) for their excellent technical assistance. The continuing support of this research project by Dr Paul J. Mohacsi is gratefully acknowledged, and we also thank Prof Urs E. Nydegger for valuable discussions.

#### REFERENCES

1. Kimberly RP, Salmon JE, Bussell JB, Crow MK, Hilgartner MW: Modulation of mononuclear phagocyte function by intravenous gamma-globulin. *J Immunol* 132:745, 1984
2. Kazatchkine MD, Rossi F, Sultan Y, Nydegger U: Anti-idiotypes against autoantibodies to procoagulant factor VIII (VIII:C) in intravenous immunoglobulins. *Intern Rev Immunol* 5:157, 1989
3. Andersson U, Bjork L, Skansen-Saphir U, Andersson J: Pooled human IgG modulates cytokine production in lymphocytes and monocytes. *Immunol Rev* 139:21, 1994
4. Ross C, Svenson M, Nielsen H, Lundsgaard C, Hansen MB, Bendtzen K: Increased in vivo antibody activity against interferon alpha, interleukin-1 alpha, and interleukin-6 after high-dose Ig therapy. *Blood* 90:2376, 1997
5. Basta M, Kirshbom P, Frank MM, Fries LF: Mechanism of therapeutic effect of high-dose intravenous immunoglobulin. Attenuation of acute, complement-dependent immune damage in a guinea pig model. *J Clin Invest* 84:1974, 1989
6. Basta M, Fries LF, Frank MM: High doses of intravenous Ig inhibit in vitro uptake of C4 fragments onto sensitized erythrocytes. *Blood* 77:376, 1991
7. Basta M, Dalakas MC: High-dose intravenous immunoglobulin exerts its beneficial effect in patients with dermatomyositis by blocking endomysial deposition of activated complement fragments. *J Clin Invest* 94:1729, 1994
8. Mollnes TE, Hogasen K, Hoaas BF, Michaelsen TE, Garred P, Harboe M: Inhibition of complement-mediated red cell lysis by immunoglobulins is dependent on the Ig isotype and its C1 binding properties. *Scand J Immunol* 41:449, 1995
9. Lutz HU, Stammler P, Jelezarova E, Nater M, Spath PJ: High doses of immunoglobulin G attenuate immune aggregate-mediated complement activation by enhancing physiologic cleavage of C3b in C3b(n)-IgG complexes. *Blood* 88:184, 1996
10. Miletic VD, Hester CG, Frank MM: Regulation of complement activity by immunoglobulin. 1. Effect of immunoglobulin isotype on C4 uptake on antibody-sensitized sheep erythrocytes and solid phase immune complexes. *J Immunol* 156:749, 1996
11. van den Berg TK, Dopp EA, Daha MR, Kraal G, Dijkstra CD: Selective inhibition of immune complex trapping by follicular dendritic cells with monoclonal antibodies against rat C3. *Eur J Immunol* 22:957, 1992
12. Tenner AJ, Lesavre PH, Cooper NR: Purification and radiolabeling of human C1q. *J Immunol* 127:648, 1981
13. Bagchus WM, Hoedemaeker PJ, Rozing J, Bakker WW: Glomerulonephritis induced by monoclonal anti-Thy 1.1 antibodies. A sequential histological and ultrastructural study in the rat. *Lab Invest* 55:680, 1986
14. Schulze M, Baker PJ, Perkinson DT, Johnson RJ, Ochi RF, Stahl RA, Couser WG: Increased urinary excretion of C5b-9 distinguishes passive Heymann nephritis in the rat. *Kidney Int* 35:60, 1989
15. Hiemstra PS, Langelier E, Compier B, Keepers Y, Leijh PC, van den Barselaar MT, Overbosch D, Daha MR: Complete and partial deficiencies of complement factor D in a Dutch family. *J Clin Invest* 84:1957, 1989
16. van den Dobbelen ME, van der Woude FJ, Schroeijers WE, van Es LA, Daha MR: Soluble aggregates of IgG and immune complexes enhance IL-6 production by renal mesangial cells. *Kidney Int* 43:544, 1993
17. Hirt W, Nebe T, Birr C: Phagotest and Bursttest (Phagoburst), test kits for study of phagocyte functions. *Wien Klin Wochenschr* 106:250, 1994
18. Basta M, Fries LF, Frank MM: High doses of intravenous immunoglobulin do not affect the recognition phase of the classical complement pathway. *Blood* 78:700, 1991
19. van Dixhoorn MGA, Gorter A, Sato T, van der Wal AM, van Eendenburg JDH, Rozing J, Daha MR, Deheer E: Induction of microhematuria by an IgA isotype switch variant of a monoclonal anti-Thy-1.1 antibody in the rat. *Kidney Int* 50:1612, 1996
20. Wagner E, Platt JL, Frank MM: High dose intravenous immunoglobulin does not affect complement-bacteria interactions. *J Immunol* 160:1936, 1998
21. Garbet ND, Munro CS, Cole PJ: Opsonic activity of a new intravenous immunoglobulin preparation: Pentaglobin compared with Sandoglobulin. *Clin Exp Immunol* 76:8, 1989
22. Poynton CH, Jackson S, Fegan C, Barnes RA, Whittaker JA: Use of IgM enriched intravenous immunoglobulin (Pentaglobin) in bone marrow transplantation. *Bone Marrow Transplant* 9:451, 1992
23. Jackson SK, Parton J, Barnes RA, Poynton CH, Fegan C: Effect of IgM-enriched intravenous immunoglobulin (Pentaglobin) on endotoxaemia and anti-endotoxin antibodies in bone marrow transplantation. *Eur J Clin Invest* 23:540, 1993

24. Rommelsheim K: Preventive use of Pentaglobin in intensive care treatment of trauma patients. *Anesthesiol Intensivmed Notfallmed Schmerzther* 24:162, 1989
25. Behre G, Ostermann H, Schedel I, Helmerking M, Schiel X, Rothenburger M, Geiger S, Dedroogh M, Bockelmann D, Wörmann B, Kienast J, Hiddemann W: Endotoxin concentrations and therapy with polyclonal IgM-enriched immunoglobulins in neutropenic cancer patients with sepsis syndrome: Pilot study and interim analysis of a randomized trial. *Antiinfect Drugs Chemother* 13:129, 1995
26. Schedel I, Dreikhausen U, Nentwig B, Hockenschneider M, Rauthmann D, Balıkcıođlu S, Coldewey R, Deicher H: Treatment of gram-negative septic shock with an immunoglobulin preparation: A prospective, randomized clinical trial. *Crit Care Med* 19:1104, 1991
27. Behre G, Schedel I, Nentwig B, Wörmann B, Essink M, Hiddemann W: Endotoxin concentration in neutropenic patients with suspected gram-negative sepsis: Correlation with clinical outcome and determination of anti-endotoxin core antibodies during therapy with polyclonal immunoglobulin M-enriched immunoglobulins. *Antimicrob Agents Chemother* 36:2139, 1992
28. Kojima T, Dalcarpio CA, Hayashi S, Yokoyama I, Takagi H: Inhibition of complement-mediated immune hemolysis by the constant domain of immunoglobulin. *Xenotransplantation* 4:75, 1997