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Modulation of alloimmune response *in vitro* by an IgM-enriched immunoglobulin preparation (Pentaglobin)

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SUMMARY

The mode of action of intravenous immunoglobulins (IVIG) in autoimmune and immunoregulatory disorders is still poorly understood. *In vitro*, direct effects of IVIG on cytokine release and on cytokine receptors have been described, as well as naturally occurring, neutralizing anti-cytokine antibodies. The aim of our study was to investigate whether the enrichment in IgM and IgA would have any impact on the *in vitro* immunomodilatory capacity of IVIG. The preparation tested (Pentaglobin) contains 76% IgG and 12% IgM and IgA, respectively. We could demonstrate a significant inhibition of alloantigen-induced proliferation in the mixed lymphocyte reaction (MLR) even at a Pentaglobin concentration of 1·0 mg/ml. About 10-fold higher concentrations of standard 7S IVIG containing only trace amounts of IgM and IgA were necessary to achieve equivalent suppression of the alloimmune response. Similarly, phytohaemagglutinin (PHA)-induced lymphocyte proliferation was more effectively inhibited by Pentaglobin than by standard 7S IVIG. Cytokine analyses in culture supernatants of MLR provide evidence that Pentaglobin not only modulates interleukin-2 (IL-2), which has already been observed with standard 7S IVIG, but, moreover, modulates interferon-γ production with a subsequent impact on monocyte-derived tumour necrosis factor-α and IL-6 release. Based on these results we conclude that *in vitro* the IgM- and IgA-enriched Pentaglobin has a more potent immunomodulatory capacity than conventionally used standard 7S IVIG.

INTRODUCTION

An immunomodulatory role of intravenous immunoglobulins (IVIG) has been suggested by reports of successful treatment of immunoregulatory and autoimmune diseases.[1–4] Using several *in vitro* models, including lectin stimulation and mixed lymphocyte reaction (MLR), we and others have demonstrated that commercially available 7S IVIG preparations interfere with activation and proliferation of immune cells as one mode of action.[5–8] Reduced interleukin-2 (IL-2) protein levels in culture supernatants of IVIG-supplemented MLR as well as the finding of reduced intracellular IL-2 protein levels in peripheral blood mononuclear cells (PBMC) stimulated by lectins in the presence of IVIG, strongly argue in favour of a post-transcriptional interference of IVIG with IL-2 production resulting in the observed diminished proliferative capacity.[5,6] Furthermore, naturally occurring anti-cytokine antibodies have been identified in various 7S IVIG preparations.[9–12] Taken together these findings suppose that cytokine modulation by IVIG might be, at least in part, responsible for the benefits observed in human bone marrow or solid organ transplant recipients receiving IVIG.[13,14] Pentaglobin is a commercially available IVIG specifically enriched in IgM and IgA and, moreover, contains toxin-binding and neutralizing antibodies to various Gram-positive and Gram-negative bacteria, such as *Escherichia coli*, *Pseudomonas* and *Klebsiella*.[15,16] Whether this IVIG preparation has, consequently, a more potent immunomodulatory capacity, not only by reducing endotoxin serum levels but also by direct effects of the IgM and/or IgA component on cellular and cytokine-mediated immune mechanisms, remains to be shown. Preliminary results of two small trials in human bone marrow transplant recipients show beneficial effects of Pentaglobin on the clinical course of acute graft-versus-host disease (GVHD) and/or infectious or endotoxin-mediated complications.[17,18] We therefore analysed the effects of Pentaglobin on T-cell activation/proliferation and cytokine production during alloimmune response *in vitro*.

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Abbreviations: BMT, bone marrow transplantation, GVHD, graft-versus-host disease; IFN, interferon; IL, interleukin; IVIG, intravenous immunoglobulin; MLR; mixed lymphocyte reaction; PHA, phytohaemagglutinin; TNF, tumour necrosis factor.

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MATERIALS AND METHODS

Cell preparations

PBMC were isolated from heparinized venous blood samples from healthy volunteers using Ficoll–Isopaque (Lymphoprep,

Nycomed, Oslo, Norway) gradient centrifugation (400 *g* for 30 min at room temperature). Cells were washed three times and then resuspended at a concentration of 1×10^6/ml in tissue culture medium.

Tissue culture medium

RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated human pool serum, 125 U/ml penicillin (Biochemie Comp., Vienna, Austria), 125 μg/ml streptomycin sulphate (Biochemie Comp), and 1·15 μg/ml amphotericin B (Squibb Pharma, Vienna, Austria) was used as tissue culture medium.

Immunoglobulin preparation

Pentaglobin (Biotest, Frankfurt, Germany) was kindly provided. The standard preparation (50 mg/ml protein) for intravenous use contains 38 mg/ml IgG (76%), 6 mg/ml IgM (12%) and 6 mg/ml IgA (12%). Stock solutions (30 mg/ml protein) were prepared with tissue culture medium, dialysed in HEPES buffer/NaCl 0·9% (1/50 v/v) and kept at −20° until use. Pentaglobin was added to the experiments after adjustment to the appropriate concentrations in 50 μl culture medium.

Intact immunoglobulins (7S IVIG, Gamimune N, Cutter Biological, Berkeley, CA) containing >99% monomeric IgG, 0·27 mg/ml IgA and 0·1 mg/ml IgM were kindly provided. Stock solutions (50 mg/ml) were prepared, extensively dialysed in HEPES buffer/NaCl 0·9% (1/50 v/v) and kept at −20° until use. Immunoglobulins were supplemented to the cultures after adjustment to appropriate concentrations in 50 μl culture medium.

Phytohaemagglutinin (PHA) stimulation

PBMC (5×10^4) were incubated in triplicate in 96-well U-bottom microtitre plates (Falcon, Becton and Dickinson, Lincoln Park, NJ) with graded doses of Pentaglobin and 1% PHA (Difco, Detroit, MI) in a humidified 5% CO_2/95% air atmosphere at 37° for 72 h. Each well was pulsed with 2 μCi of [^{3}H]thymidine (40–60 Ci/mmol, Amersham, Arlington Heights, IL) for the last 16 h of culture. Cells were harvested using a semiautomatic device, and [^{3}H]thymidine uptake was measured in a liquid scintillation counter. Results are expressed as mean counts per minute (c.p.m.) of triplicate cultures ± standard error of the means (SEM).

Mixed lymphocyte reaction (MLR)

Proliferative response of PBMC to allogeneic stimulation was determined ina one-way MLR, as previously described.[19] Different responder/stimulator combinations were used for each experiment. Responder PBMC (5×10^4) were co-cultured with 5×10^4 of either mitomycin C-treated (Sigma, Munich, Germany) allogeneic PBMC or mitomycin C-treated autologous PBMC. Pentaglobin was added at the initiation of cultures in appropriate concentrations to give a final volume of 150 μl/well. Culture plates were incubated for 6 days at 37° in a humidified 5% CO_2/95% air atmosphere and exposed to 2 μCi/well of [^{3}H]thymidine for the last 16 hr of culture. Harvesting and [^{3}H]thymidine uptake measurements were performed as described and c.p.m. were given as mean of triplicates ± SEM.

Cytokine determination

Culture supernatants of MLR were analysed for cytokines and soluble IL-2 receptor (sIL-2R) levels at the start and after 72 and 144 hr of culture using commercially available kits [tumour necrosis factor-α] [(TNF-α, IL-6, interferon-γ (IFN-γ), by radioimmunoassay (RIA); IRE-Medgenix, Brussels, Belgium; sIL-2R, by enzyme linked immunosorbent assay, ELISA; Immunotech S. A. Marseille, France)].

Minimum detection levels were 3 pg/ml for TNF-α, 3 pg/ml for IL-6, 0·01 IU/ml for IFN-γ and 0·3 ng/ml for sIL-2R.

IL-2 bioassay

IL-2 activity in culture supernatants from day 0 and 3 of MLR was determined by its ability to induce proliferation of the IL-2-dependent CTLL-16 cell line as previously described.[5] Briefly, $1{\cdot}5 \times 10^4$/25 μl CTLL-16 cells (kindly provided by Dr K. Heeg, Ulm, Germany) were incubated in round-bottom microtitre plates with 75 μl of supernatant or serial dilutions of *E. coli*-derived recombinant human IL-2 (rhIL-2; Proleukin, Laevosan GMBH, Linz, Austria) as standard to give a final volume of 100 μl/well. After 24 hr of incubation each well was pulsed with 2 μCi of [^{3}H]thymidine (40–60 Ci/mmol, Amersham, Arlington Heights, IL, USA) for another 16 hours of culture. Cells were harvested using a semiautomatic device, and [^{3}H]thymidine uptake was measured as described above. Half maximal proliferation of CTLL-16 cells with rhIL-2 was achieved at a concentration of 0·4 IU/ml.

Statistical analysis

Differences in mean values were computed for their statistical significance using the Student's two-tailed *t*-test.

RESULTS

Effects of Pentaglobin on PHA stimulation and MLR

Proliferation of PHA-stimulated PBMC was dose-dependently suppressed to 41 ± 10% of the control by supplementation of 10 mg/ml Pentaglobin to the cultures (Fig. 1). On a gram

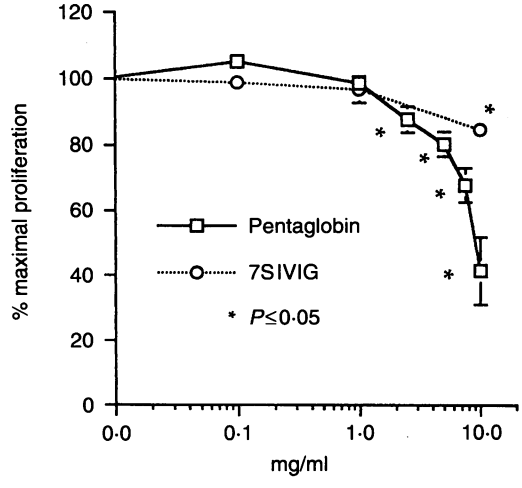

Figure 1. Effects of Pentaglobin compared to 7S IVIG (Gamimune N, Cutter Biological, Berkeley, CA) on PHA stimulation of PBMC. Mean values ± SEM of 11 different experiments are given. Absolute c.p.m. without Pentaglobin were 155 598 ± 37 969 and were set at 100%.

basis, Pentaglobin was more effective than standard 7S IVIG. Cell viability as assessed by eosin dye staining was not affected by either immunoglobulin preparation.

Addition of Pentaglobin to MLR resulted in a significant and dose-dependent suppression of proliferation to 34±3% of the control even at a concentration of 1·0 mg/ml. Tenfold higher concentrations of 7S IVIG were necessary to achieve equivalent suppression of MLR. At a concentration of 10 mg/ml the proliferation in the MLR was completely blocked by Pentaglobin but not by 7S IVIG (Fig. 2).

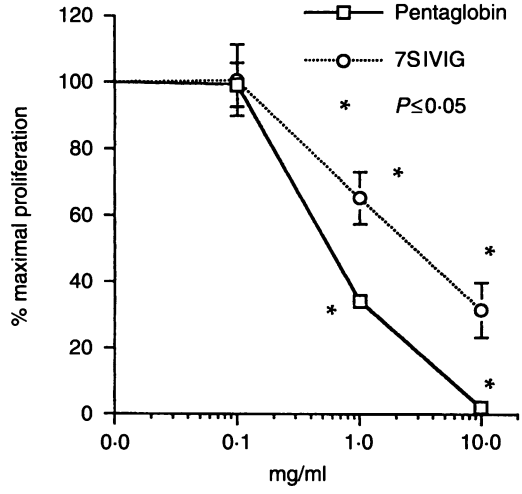

Figure 2. Effects of Pentaglobin compared to 7S IVIG on MLR. Mean values±SEM of ten different experiments using ten different responder stimulator combinations are given. Absolute c.p.m. without Pentaglobin were 37 350±4807 and were set at 100%.

Effects of Pentaglobin on cytokine and cytokine antagonist production during MLR

Cytokines were analysed in culture supernatants at the start, and after 72 and 144 hr, using commercially available kits or bioassay. Results are summarized in Table 1 and Fig. 3. Pentaglobin and 7S IVIG both significantly and dose-dependently reduced the IL-2 bioactivity in MLR culture supernatants (Fig. 3). By analysing supernatants of day 3 of

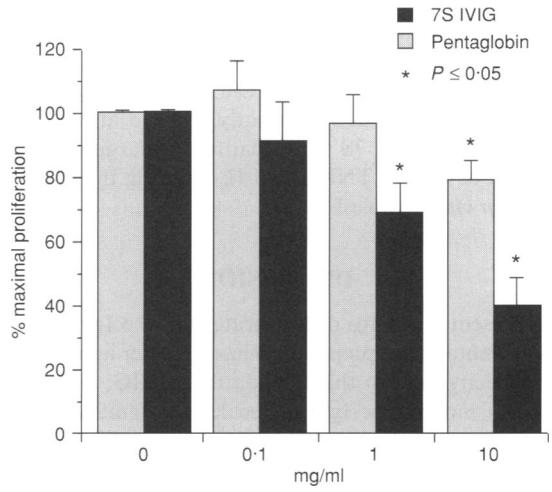

Figure 3. IL-2 bioactivity on day 3 of a 6-day MLR, determined by CTLL-16 proliferation, was significantly reduced in culture supernatants of Pentaglobin- and 7S IVIG-treated MLR (mean values±SEM of five experiments are given). CTLL-16 proliferation in the presence of supernatants from unmanipulated MLR was 3066±255 c.p.m. and was set at 100%. Basal proliferation was 317±39 c.p.m.

Table 1. Effects of Pentaglobin on cytokine protein levels in MLR supernatants. Results of five experiments using five different reponder/stimulator combinations are given as normalized mean values±SEM. Absolute protein levels for IFN-γ at 72 hr and 144 hr were 3.3±2.1 IU/ml and 9.2±3.1 IU/ml, for TNF-α 65.0±23.8 pg/ml and 233.4±70.2 pg/ml, and for IL-6 133±28 pg/ml and 394±183 pg/ml, respectively, and were set 1.0

Cytokine	Immunoglobulin (mg/ml)	72 hr		144 hr	
		Pentaglobin	7S IVIG	Pentaglobin	7S IVIG
IFN-γ	0	1·00	1·00	1·00	1·00
	0·01	2·95±0·68*	0·76±0·14	2·09±0·49	0·94±0·13
	0·1	4·80±1·45	1·08±0·34	3·82±1·86	0·96±0·20
	1	2·77±0·96	0·52±0·06*	1·42±0·81	0·60±0·15
	10	1·39±0·86	0·92±0·37	0·54±0·49*	0·90±0·41
TNF-α	0	1·00	1·00	1·00	1·00
	0·01	5·46±0·94*	0·57±0·27	2·60±0·52*	0·79±0·10
	0·1	28·45±4·02*	0·61±0·26	9·11±2·99*	0·82±0·09
	1	26·54±4·44*	0·70±0·32	7·32±2·44*	0·88±0·25
	10	0·86±0·25	3·97±1·87	0·16±0·07*	2·87±1·03
IL-6	0	1·00	1·00	1·00	1·00
	0·01	3·60±0·88*	n.d.	2·94±1·13	n.d.
	0·1	12·18±5·96	n.d.	10·12±5·20	n.d.
	1	8·49±5·37	n.d.	6·10±4·61	n.d.
	10	0·08±0·04*	1·04±0·42	0·05±0·02*	0·74±0·31

*$P \leq 0{\cdot}05$ (*t*-test)

MLR cultures, IL-2 bioactivity determined by proliferation of the IL-2 dependent CTLL-16 cell line was reduced to 74±3% and 40±9% of the control cultures if the MLR was supplemented with 10 mg/ml Pentaglobin or 7S IVIG, respectively. The reduction of IL-2 levels during alloimmune response *in vitro* by Pentaglobin and 7S IVIG was not due to an induction of sIL-2R (data not shown). Furthermore, Pentaglobin exhibited a dose-dependent, bidirectional effect on IFN-γ release. In detail, addition of Pentaglobin to MLR at concentrations ≤0·1 mg/ml stimulated IFN-γ, whereas supplementation of higher Pentaglobin concentrations to the cultures resulted in a significant reduction of IFN-γ protein levels after 6 days of culture. Similarly, TNF-α and IL-6 were both stimulated at low concentrations, but significantly reduced at high concentrations. In contrast, 7S IVIG had only marginal effects on the release of IFN-γ, TNF-α and IL-6 during the alloimmune response *in vitro* (Table 1).

DISCUSSION

In the present study we demonstrate that the IgM- and IgA-enriched Pentaglobin preparation has a greater immunomodulatory capacity *in vitro* than standard 7S IVIG, which mainly consists of monomeric IgG molecules. A significant suppression of alloantigen-induced T-lymphocyte proliferation in the MLR was achieved even at a concentration of 1 mg/ml. About tenfold higher concentrations of standard 7S IVIG were necessary for similar effects, suggesting additional immunomodulatory mechanisms mediated by either the IgA and/or IgM component of the preparation. However, whether direct interactions of IgA or IgM with immune cells, or modulation of cytokine-mediated immune reactions are involved in this context is as yet unknown.

The mechanisms of IVIG activity have been extensively studied during the past years. We have recently shown that IVIG down-regulates IL-2 protein production, resulting in a significant inhibition of human T-lymphocyte alloproliferative response *in vitro*.[5] Reports by at least two other groups confirm our observations by showing inhibitory effects of standard 7S IVIG on cytokine-dependent T-lymphocyte proliferation *in vitro* as well as reduced intracellular IL-2 protein levels in lectin-stimulated PBMC.[6,7] Here we demonstrate, that Pentaglobin in addition to a modulation of IL-2 exhibited differential effects on the release of IFN-γ, TNF-α and IL-6 during MLR response. Although Toungouz *et al.* using a bidirectional MLR system recently described an inhibition of proliferation and TNF-α production due to IFN-γ blockade by IVIG we had not found a significant modulation of cytokines other than IL-2 by standard 7S IVIG using a one-way MLR system.[5,9] Also Andersson *et al.* did not see an effect of standard 7S IVIG on IFN-γ and TNF-α production in phorbolmyristate acetate (PMA)/ionomycin-stimulated cultures of PBMC but a significant inhibition of both cytokines in anti-CD3-stimulated cultures probably indicating an inducer-specific effect of IVIG on *in vitro* cytokine production.[20]

Neutralization or blocking of cytokines by natural anti-cytokine antibodies or induction of cytokine antagonists have also been proposed to account for the immunomodulatory capacity of 7S IVIG but their relevance for IVIG activity *in vivo* remains a matter of debate.[10–12,21,22] However, due to the stimulatory effects on the release of IFN-γ, TNF-α and IL-6 at low Pentaglobin concentrations and the lack of a significant induction of sIL-2R by either 7S IVIG or Pentaglobin during MLR, it is very unlikely that anti-cytokine antibodies are indeed involved in the mechanism by which Pentaglobin or 7S IVIG acts during alloimmune response *in vitro* [5].

Nonetheless, the modulation, not only of IL-2, but also of IFN-γ and TNF-α may have further implications on the clinical usefulness of Pentaglobin in the prevention and treatment of GVHD or other immunoregulatory/autoimmune diseases. Increased serum levels of IFN-γ are detectable in patients suffering from acute GVHD after bone marrow transplant (BMT) and increased serum levels of TNF-α were associated with a higher probability of transplant-related mortality.[23,24] Modulation of these cytokines might therefore ameliorate GVHD and might have been responsible for the improvement of already established acute GVHD in 5/8 five of eight bone marrow transplant recipients treated with high-dose Pentaglobin.[17] Whether the stimulatory effects on these cytokines observed *in vitro* at low Pentaglobin concentrations have any impact on possible side-effects of IVIG treatment *in vivo* or on cytokine-mediated toxicities after bone marrow transplant such as veno-occlusive disease (VOD) remains controversial. Klaesson *et al.* reported a significantly higher death rate due to severe VOD in patients receiving high-dose 7S IVIG weekly after allogeneic (BMT) as compared to transplant recipients without such treatment.[25] Whether this phenomenon is merely the result of an increased plasma viscosity due to IVIG infusion as discussed by the authors or is rather caused by a modulation of cytokines, which have been shown to play a role in the pathogenesis of VOD[26] is unknown. In contrast to these findings, an earlier report described a beneficial effect of the IgM- and IgA-enriched Pentaglobin in BMT recipients by reducing hepatic toxicity.[18] It has to be shown whether these findings *in vivo* only result from significantly reduced endotoxin levels in Pentaglobin-treated patients or from cytokine modulation by Pentaglobin as supposed from our results or both.

We conclude that *in vitro*, Pentaglobin at concentrations readily achieved *in vivo*[17] has a greater immunomodulatory capacity as compared to standard 7S IVIG. Modulation of immune cell activation and cytokines, such as IFN-γ, TNF-α and IL-6 in addition to IL-2, together with the recently published data on neutralizing anti-idiotypes against a variety of disease-causing autoantibodies in pooled normal human polyspecific IgM and their protective effect against experimental autoimmune disease justify further studies for the development of new preventive and therapeutic protocols that take advantage of Pentaglobin (or IgM)-mediated immunosuppression in immunoregulatory disorders and bone marrow transplant recipients.[27]

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