Characterization of B cell responses in relation to organ transplantation

Heidt, S.

Chapter 4

Intravenous immunoglobulin preparations have no direct effect on B cell proliferation and immunoglobulin production

Sebastiaan Heidt, Dave L. Roelen, Chantal Eijsink, Michael Eikmans, Frans H.J. Claas and Arend Mulder

Clinical and Experimental Immunology 2009; 158 (I): 99-105

ABSTRACT

Intravenous immunoglobulin (IVIg) is used for treatment of a variety of immunological disorders and in transplantation. As one of its applications in transplantation is the reduction of donor specific antibodies in the circulation, we examined the direct effect of IVIg on essential parameters of human B cell responses *in vitro*. Purified human B cells, human B cell hybridomas and T cells were cultured in the presence of graded concentrations of IVIg to test its effect on their proliferative capacity. To address the effect of IVIg on immunoglobulin production, we designed a novel technique making use of quantitative PCR to assess IgM and IgG levels. IVIg failed to inhibit proliferation of human B cells and human B cell hybridomas. In contrast, when IVIg was added to T cell cultures, a dose-dependent reduction of the proliferative capacity was observed. IVIg did not to affect the levels of IgM and IgG mRNA of activated B cells. Our data show that IVIg is not capable of directly inhibiting key B cell responses. Direct B cell inhibition by IVIg seems therefore unlikely, implying that alteration in humoral immunity by IVIg is due to indirect effects on T cells and/or interactions with circulating antibodies and complement factors.

INTRODUCTION

Intravenous immunoglobulin (IVIg) is a preparation of pooled human plasma prepared from over a thousand donors and was originally used as a substitution therapy for patients with immunodeficiencies (*1*). IVIg is increasingly used as treatment for autoimmune and systemic inflammatory diseases, and in the field of solid organ- and bone marrow transplantation. In organ transplantation settings, IVIg is used, among other treatments, to reduce plasma HLA antibody levels prior to transplantation (*2-4*) and in attempts to reverse acute humoral rejection (*5*).

The present study focuses on the effect of IVIg on humoral immunity. Several mechanisms have been proposed for its mode of action. Firstly, anti-idiotypic antibodies in IVIg may interfere in the reaction of HLA-specific antibodies with their targets on HLA mismatched organs, thereby preventing humoral rejection (*6*). Secondly, IVIg may bind through the Fc portion of IgG to the inhibitory FcγRIIb expressed on a variety of blood cells, including B cells, leading to the reduction of proliferation and apoptosis induction (*7*). Thirdly, IVIg may inhibit complement, and thereby modulate the effector function of antibodies (*8*). Fourthly, changes in monocyte Fc receptor functions can inhibit antibody-mediated immune damage (*9*). Lastly, clearance of pathogenic antibodies may be accelerated by saturation of the FcRn receptor (*10*).

We used a robust culture system, devoid of auxiliary cells, for stimulating purified human B cells, as well as autonomously proliferating human B cell hybridomas to study the direct effect of IVIg on B cell proliferation and immunoglobulin production. The latter was assessed by a novel, PCR based, technique for quantifying immunoglobulin production.

MATERIALS AND METHODS

Cells

Blood was collected from healthy blood bank donors with informed consent under guidelines issued by the Medical Ethics Committee of the Leiden University Medical Center (Leiden, the Netherlands). Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Hypaque density gradient centrifugation. B cells were immunomagnetically isolated from PBMC by positive selection using Dynabeads CD19 pan B (Invitrogen, Leek, the Netherlands) and released from the beads using Detach-a-Bead CD19 (Invitrogen). This yielded >98% pure B cells, as assessed by flow cytometric analysis. Human B cell hybridomas producing a variety of HLA monoclonal antibodies (mAbs) were established

as described previously (*11*). The hybridomas used were SN607D8 (HLA-A2/A28, IgG1), BVK5B10 (HLA-B8, IgM), VTM1F11 (HLA-B27/B7/B60, IgG1) and SN66E3 (HLA-A2/A28, IgM).

T cells were purified from PBMC by magnetic separation using the Pan T cell isolation kit II (Miltenyi, Bergisch-Gladbach, Germany) and MACS MS columns (Miltenyi). Flow cytometric analysis revealed >85% purity.

Proliferation experiments

B cells were cultured for 7 days at 5×10^3 cells/well in 96-well roundbottom plates (BD Falcon, Breda, the Netherlands) in culture medium consisting of IMDM (Gibco, Paisley, UK) supplemented with 10% FCS (Gibco), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, the Netherlands) and ITS (insulin 5 µg/ml, transferrin 5 µg/ml and selenium 5 ng/ml, Sigma-Aldrich) at 37°C and 5% CO_2. B cells were stimulated with anti-CD40 mAb (1 µg/ml, R&D systems, Minneapolis, MN, USA), 100 U/ml IL-2 (EuroCetus, Amsterdam, the Netherlands), 25 ng/ml IL-10 (R&D Systems), 100 ng/ml IL-21 (Invitrogen) and 2.5 µg/ml of the TLR9 ligand CpG ODN 2006 (Hycult Biotechnology, Uden, the Netherlands). T cells were stimulated with 1 µg/ml phytogaemagglutinin (PHA, Remel, Lenexa, KS, USA) and cultured at 10^4 cells/well for 3 days in culture medium without ITS. Autonomously growing B cell hybridomas were cultured at 10^3 cells/well for 5 days in IMDM with 5% FCS, 0.05 mM 2-mercaptoethanol and 2 mM L-glycyl-L-glutamine (Sigma-Aldrich).

All cultures were performed in the absence or presence of graded concentrations of IVIg (Sanquin, Amsterdam, the Netherlands). Multiple lot numbers of IVIg were used, giving comparable results. Proliferation experiments were also performed with Gammagard (Baxter, Utrecht, the Netherlands), which gave similar results. Physical properties of the IVIg preparations are shown in Table 1. All data shown were obtained with IVIg from Sanquin.

Table 1. *Physical properties of IVIg preparations used.*

Product	Manufacturer	Method of preparation	% IgG	Stabilizing agent
Immunoglobulin i.v.	Sanquin	Cohn's cold ethanol fractionation	> 95%	Glucose
Gammagard S/D	Baxter	Cohn-Oncley-fractionation, ion-exchange, ultrafiltration, chromatography	> 90%	Glucose, glycine, albumin, PEG

i.v.: intravenous, PEG: polyethylene glycol.

All IVIg preparations were dialyzed prior to use unless otherwise stated. Dialysis was performed in 10 kDa Slide-A-Lyzer cassettes (Pierce, Rockford, IL, USA) against large volumes of IMDM. The presence of intact IgG after dialysis was confirmed by ELISA (data not shown). Where appropriate, cell culture grade BSA (Calbiochem, La Jolla, CA, USA) was used as a control for the high protein concentrations and D(+) glucose (Merck, Amsterdam, the Netherlands) was used as a control for the stabilizing agent of IVIg. Proliferation was determined by the incorporation of ^{3}H-TdR (1 µCi/well, Amersham International, Amersham, UK), added 18 hours before termination of culture.

Quantitative PCR

For IgM and IgG mRNA detection, B cells were cultured at 5×10^{5} cells per well for 7 days in 24-well plates (Costar, Veenendaal, the Netherlands) and stimulated as described above in the presence of graded concentrations of IVIg. Rapamycin was used as a control for the inhibition of immunoglobulin production (*12*). Cells were harvested and preserved in RNAlater solution (Qiagen, Chatsworth, CA, USA). RNA was extracted using the RNeasy® mini kit (Qiagen) following the manufacturer's instructions. RNA was treated with DNase (Qiagen) on the spin columns. RNA quantity was assessed with a spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). All samples showed A260/A280 ratios between 1.9 and 2.1. cDNA was synthesized by incubating 12.8 µl RNA solution with 7.2 µl cDNA mix containing dNTPs (final concentration of 0.5 mM), 2 U reverse transcriptase-avian myeloblatosis virus (RT-AMV), 20 U rRNase inhibitor, 100 ng oligo-dT primers, 500 ng random primers, and 1× reverse transcriptase buffer (all from Promega, Leiden, the Netherlands).

Primer sets (Table 2) for quantitative polymerase chain reaction (Q-PCR) were selected using Beacon Designer Software (version 7.02, Premier Biosoft International, Palo Alto, CA, USA) and obtained from Eurogentec (Liège, Belgium). For the amplification of total IgG transcript two forward primers, one hybridizing to the IgG_1 sequence and the other to IgG_2, IgG_3 and IgG_4 sequences, were used at a 1:1 ratio. The IgG reverse primer detected all four IgG variants. PCR mixes contained 1 µM of forward and reverse primers, 3 mM $MgCl_2$, and 1× iQ SYBR Green supermix (Bio-Rad, Veenendaal, the Netherlands). The PCR was performed using an iCycler MyiQ (Bio-Rad). The PCR program consisted of one cycle of 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C and was finalized with a melting curve analysis. Reactions were carried out in optical 96-well plates (Bio-Rad) covered with Microseal 'B' Film (Bio-Rad). The signal of the stably expressed household gene 18S rRNA served as a normalization factor.

Table 2. *Sequences for primers used in quantitative polymerase chain reaction (Q-PCR).*

Transcript	Forward primer	Reverse primer	Amplicon
IgG_1	CATCTCCAAAGCCAAAGG	ATGTCGCTGGGATAGAAG	126 bp
IgG_{2-4}	CATCTCCAAAGCCAAAGG	ATGTCGCTGGGGTAGAAG	126 bp
IgM	CAGGGCACAGACGAACAC	CGGCAATCACTGGAAGAGG	85 bp
18S rRNA	AGTCCCTGCCCTTTGTACACA	GATCCGAGGGCCTCACTAAAC	68 bp

bp: base pairs.

RESULTS

IVIg fails to inhibit B cell and B cell hybridoma proliferation

IVIg (at concentrations up to 35 mg/ml) failed to affect the proliferative capacity of both purified *in vitro* stimulated B cells from different donors (n=5) as well as four autonomously growing B cell hybridomas (Figure 1a and 1b). In contrast, non-dialyzed IVIg did inhibit the proliferation of B cell hybridomas (Figure 1c), which is in agreement with published data (*13*). However, inhibition was not due to the immunoglobulin component, as is clear from the lack of proliferation inhibition with dialyzed IVIg, but rather caused by the stabilizing agent glucose that is present in the IVIg solution (Figure 1d).

Since IVIg has been described to inhibit mitogen-induced lymphocyte proliferation (*14*), we tested its capacity to inhibit PHA induced T cell proliferation (n=4). The IVIg preparation dose-dependently inhibited T cell proliferation, indicating that the IVIg preparation was functional (Figure 1e). This inhibition was not due to high protein concentration, as shown by the inability of comparable concentrations of BSA to inhibit proliferation (Figure 1f).

IVIg does not inhibit immunoglobulin mRNA levels

Previously, we showed that B cells cultured in the presence of anti-CD40 mAb, IL-2, IL-10, IL-21 and CpG ODN 2006 were capable of producing large quantities of both IgM and IgG (*12*). This stimulation protocol induced vast elevations in mRNA levels for both IgM and IgG when compared to unstimulated B cells (Figure 2a and 2b), which is in concordance with immunoglobulin levels measured in the supernatants of these B cell cultures by ELISA (data not shown).

To test the effect of IVIg on immunoglobulin production, B cells were stimulated in the presence of IVIg at graded concentrations up to 35 mg/ml. Rapamycin was used as positive control for the reduction of immunoglobulin production. Q-PCR with primers specific for IgM and IgG revealed that IVIg did not affect the levels of IgM or IgG mRNA, whereas rapamycin dose-dependently reduced IgM and IgG mRNA levels (Figure 3a and 3b).

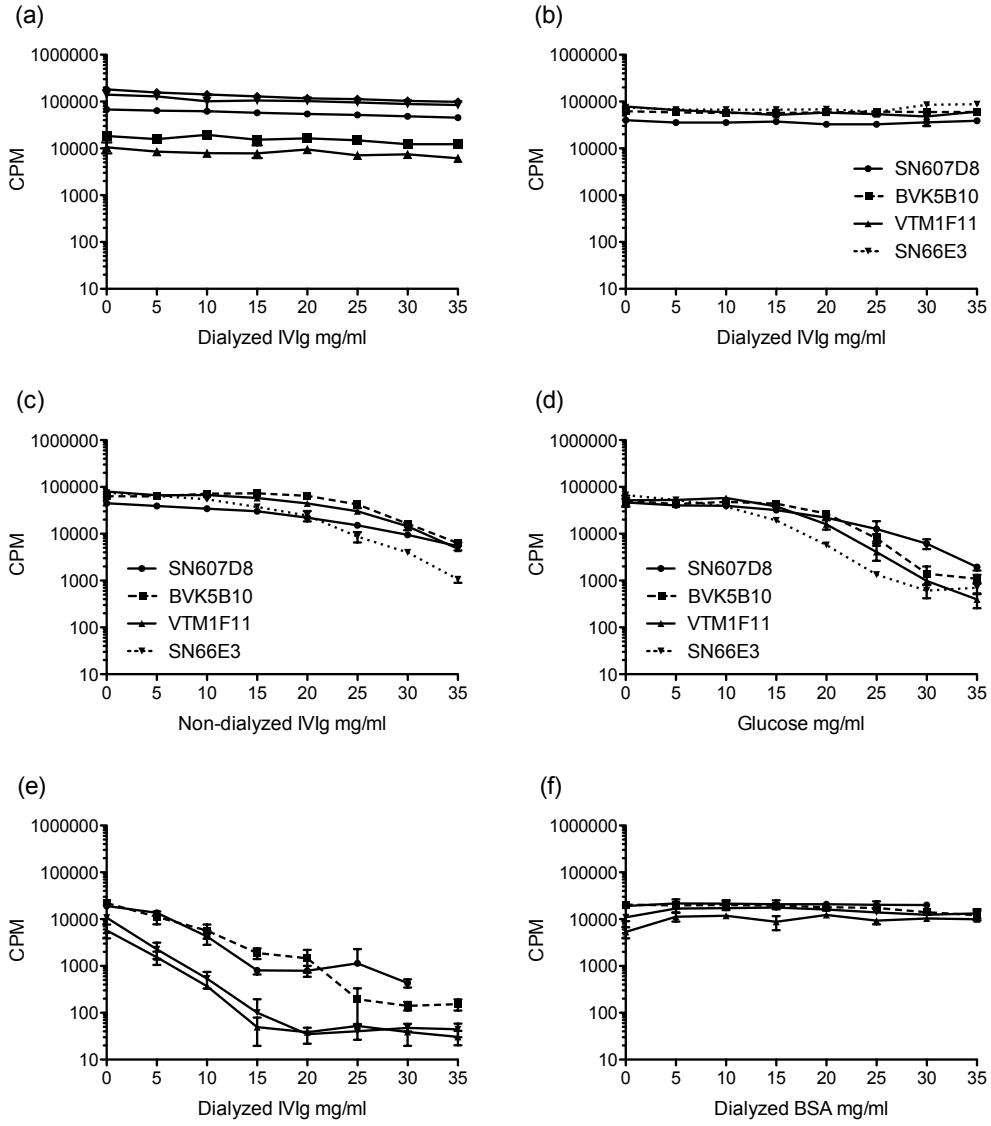

Figure 1. *IVIg does not reduce the proliferative capacity of B cells and B cell hybridomas, whereas mitogen-induced T cell proliferation is inhibited. Non-dialyzed IVIg does inhibit hybridoma proliferation, caused by the stabilizing agent glucose. (a) B cells were cultured at 5×10³ cells/well with the addition of anti-CD40 mAb, IL-2, IL-10, IL-21 and CpG ODN 2006 in the presence of graded concentrations of IVIg. Connecting lines represent the individuals donors (n=5) tested. (b) Autonomously growing B cell hybridomas were cultured at 10³ cells/well in the presence of graded concentrations of IVIg, (c) non-dialyzed IVIg or (d) glucose in concentrations similar to the IVIg solution. Connecting lines represent individual hybridomas. Representative experiments are shown. (e) T cells were isolated by MACS isolation and cultured at 10⁴ cells per well with 1 mg/ml PHA in the presence of graded concentrations of IVIg or (f) BSA in concentrations equal to the IVIg solution. Connecting lines represent the individuals donors (n=4) tested. Proliferation was measured by ³H-TdR incorporation, CPM: counts per minute. Depicted are mean values of triplicate measurements.*

DISCUSSION

IVIg is known to have a wide range of clinical effects. When used *in vitro*, IVIg reduced the reactivity of serum HLA antibodies (*15, 16*). It also inhibited mixed lymphocyte responses, and mitogen induced PBMC and T cell proliferation (*14*), as well as T cell dependent B cell responses (*17-20*).

The present study shows that IVIg does not affect the proliferative capacity of polyclonally stimulated purified B cells. This lack of inhibition is not due to extremely high B cell stimulation that is no longer amenable to interference, since we have previously shown that relatively low levels of standard immunosuppressive drugs can completely abolish proliferation, as well as immunoglobulin production of B cells stimulated with the same protocol (*12*). In contrast to the lack of B cell proliferation inhibition by IVIg, mitogen-induced T cell proliferation was significantly inhibited by the same IVIg preparation, indicating that the IVIg preparation was effective. This inhibition was not due to high protein levels, indicating that immunoglobulins present in the IVIg preparation were the cause of proliferation inhibition.

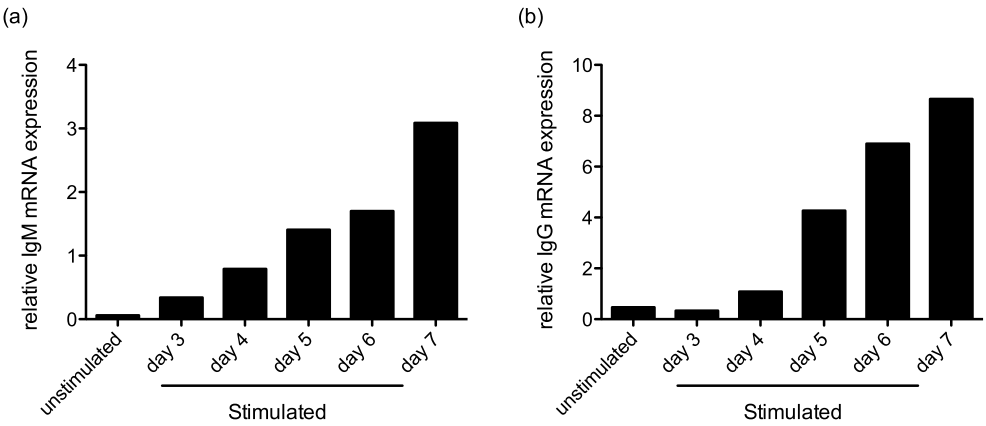

Figure 2. *B cell activation induces IgM and IgG mRNA levels. B cells were activated with anti-CD40 mAb, IL-2, IL-10, IL-21 and CpG ODN 2006 and the cells were harvested at different time-points after activation. IgM (a) and IgG (b) mRNA levels were assessed by quantitative PCR. Unstimulated cells were harvested at day 0 and served as negative control. The results of one representative experiment are shown. Similar results were obtained in 4 independent experiments.*

B cell hybridomas were previously shown to be inhibited by a non-dialyzed IVIg preparation (*13*). We added non-dialyzed IVIg to similar B cell hybridomas and observed a comparable

inhibition. However, when we dialyzed the IVIg prior to use to remove stabilizing agents such as glucose from the solution, the inhibition was abrogated. In agreement with previous studies (*21*), addition of glucose in concentrations similar to those in the IVIg preparation did inhibit the proliferation of B hybridomas. These data underscore the importance of dialysis of the IVIg preparation for *in vitro* studies.

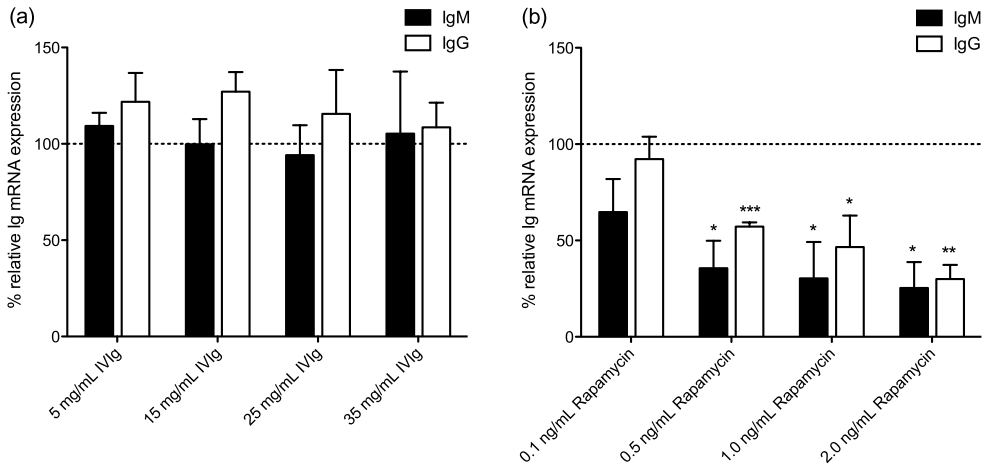

Figure 3. *IVIg does not affect the mRNA synthesis of IgM and IgG, whereas rapamycin does inhibit IgM and IgG mRNA levels. B cells were stimulated with anti-CD40 mAb, IL-2, IL-10, IL-21 and CpG ODN 2006 and cultured in the presence of graded concentrations of IVIg (a). Control cultures were performed with graded concentrations of rapamycin. mRNA levels for IgM and IgG were quantified using quantitative PCR (b). The dotted lines represent the mRNA steady state level in stimulated, non-treated B cells. Data from 3 experiments with different donors are shown. Depicted are the percentages of IgM and IgG mRNA expression, relative to the mRNA expression of untreated controls. Statistics: paired T test, *P<0.05; **P<0.01 and ***P<0.001.*

Besides B cell proliferation, studying the effects of IVIg on immunoglobulin production is of particular interest, since anti-idiotypic antibody binding and binding to inhibitory Fc receptors are candidate mechanisms for interference with immunoglobulin production (*6, 7*).

Unfortunately, the high IgG content of IVIg precludes the detection of newly synthesized IgG by standard ELISA techniques. De Grandmont *et al.* circumvented this issue by radioactive labeling of secreted immunoglobulins followed by capture with anti-IgG antibodies (*22*). But even then, competition by IVIg for capture antibody cannot be excluded. Therefore, we designed a novel approach to indirectly measure immunoglobulin production by detection of IgM and IgG mRNA levels. We found that IVIg does not affect the mRNA synthesis of

both IgM and IgG by stimulated B cells. It seems therefore highly unlikely that IVIg directly influences immunoglobulin production.

This conclusion is at variance with other studies that do show an inhibitory effect of IVIg on immunoglobulin production (*17-20*). These studies, which describe B cells as targets for IVIg, were performed with Pokeweed Mitogen stimulation, which is a model of T cell dependent antibody production by B cells (*23*). However, it cannot be ruled out that in that culture system IVIg interferes with the actual stimulus, *i.e.* Pokeweed Mitogen, rather than that it truly inhibits B cell functions. In another study, inhibition of proliferation and IgE secretion of purified tonsillar B cells cultured with IL-4 and anti-CD40 was described (*24*). However, these results were obtained using a non-dialyzed IVIg preparation, and are likely an effect of the stabilizing agents in the IVIg preparation.

IVIg preparations differ greatly in terms of production process and Ig content. While B cell inhibitory effects were not found in two different IVIg preparations, it cannot be ruled out that other preparations will show inhibitory effects, particularly when other isotypes are present, as in Pentaglobulin.

In line with our results, a series of *in vivo* studies show the lack of direct effects on immunoglobulin producing cells. IVIg was unable to reduce B cell and plasma cell numbers in human splenic follicles (*25*). Furthermore, IVIg failed to reduce the number of bone marrow residing long-lived plasma cells producing HLA-specific antibodies (*26*).

The inhibitory effect of IVIg on humoral immunity may therefore be explained by alternative mechanisms, such as inhibition of T cell help, interaction with circulating antibodies and interaction with complement factors. Inhibitory effects of IVIg on T cell priming by dendritic cells have been described (*27*), as well as cytokine-dependent T cell proliferation (*28*). Inhibition of T cell function may lead to insufficient generation of helper T cells, which affects B cell responses. Future research on the effect of IVIg on T cell help directly, and on T cell dependent B cell responses in co-culture systems will provide more insight in the effect of IVIg on humoral immune responses.

Interaction of the variable regions of IVIg with variable regions of autoantibodies of multiple specificities has been described (*29, 30*), suggesting anti-idiotypic activity of IVIg. Circulating anti-HLA antibodies may be subject to the same mechanism of suppression. In addition, interference of IVIg with complement activation (*8, 31*) and impairment of monocyte Fc receptor functions (*9*) have been suggested. These mechanisms may affect the action of deleterious complement binding antibodies and/or phagocytosis, leading to protection from immune damage. Alternatively, saturation of the FcRn receptor may be involved in clearance of pathogenic antibodies (*10*).

Taken together, our results show that IVIg fails to directly act on immunoglobulin producing

cells. The capability of IVIg to interfere with humoral immunity is therefore more likely due to effects on T cells and interactions with circulating antibodies and/or complement factors.

ACKNOWLEDGEMENTS

This work was supported by The Landsteiner Foundation for Blood Transfusion Research and the National Reference Center for Histocompatibility Testing. The authors thank Jacqueline Anholts for technical assistance in the quantitative PCR assays and Cees van Kooten and Anneke Brand for critically reading the manuscript.

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