ORIGINAL ARTICLE

ARE IgG-ANTI-FAB2$_\gamma$ AND IgG-ANTI-HINGE ANTIBODIES ACTIVE CONSTITUENTS OF INTRAVENOUS IMMUNOGLOBULIN?

DAN NAVOLAN[1#]*, IOAN SAS[1#], PETER TERNESS[2]

[1]*Department of Obstetrics-Gynaecology and Neonatology, "Victor Babes" University of Medicine and Pharmacy, 3 Odobescu Street, 300202, Timisoara, Romania*
[2]*Department of Transplantation-Immunology, Institute for Immunology, University of Heidelberg, 69120, Heidelberg, Germany*

**corresponding author: navolan@yahoo.com*
[#] *All authors have equal contribution.*

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Abstract

Our aim was to find out if immunoglobulins for intravenous use (IVIG) contain IgG-anti-F(ab')2$_\gamma$ (AFab2) and IgG-anti-Hinge (AH) antibodies (Ab.) and to discuss how AFab2, in particular AH could contribute to immunomodulatory effect of IVIGs. We measured IgG, AFab2 and AH in five IVIG products approved for therapeutic use. IVIG showed higher AFab2 and AH concentration than sera of randomized healthy controls. Individual IVIG products showed different AFab2 and AH concentrations. AFab2 and AH were thought to exert an immunosuppressive effect in patients with certain autoimmune diseases, graft recipients and pregnant women. Since in these cases the disease may be caused by a decreased AFab2 and AH concentration abrogating the immunotolerance, it is expected that administration of AFab2 and AH containing IVIGs would reverse the immunotolerance and cure the disease. The data presented herein show for the first time that some IVIGs contain a significant amount of AFab2 and AH and thus open the door for substitutive therapies of diseases in which these immunosuppressive antibodies play a pathogenetic role.

Rezumat

Scopul stuiului nostru a fost evaluarea imunoglobulinelor de uz intravenos (IVIG) asupra conținutului în anticorpi IgG-anti-F(ab')2$_\gamma$ (AFab2) și IgG-anti-Hinge (AH) și comentarea unor noi mecanisme prin care anticorpii AFab2, în particular AH pot contribui la efectul imunomodulator al IVIG. Am măsurat anticorpii IgG, AFab2 și AH în cinci produse IVIG aprobate pentru uz terapeutic. IVIG prezintă un titru de anticorpi AFab2 și AH mai mare decât serurile voluntarilor sănătoși. Anumite produse IVIG prezintă concentrații diferite de anticorpi AFab2 și AH. Se presupune că anticorpii AFab2 și AH exercită un efect imunosupresiv la pacienți cu anumite boli autoimune, pacienți transplantați și gravide. Deoarece aceste boli (boli autoimune, rejetul grefei, avortul spontan și nașterea prematură) pot fi cauzate de o scădere a titrului de anticorpi AFab2 și AH, având ca și consecință anularea imunotoleranței, este de așteptat ca administrarea de IVIG conținând AFab2 și AH sa restabilească imunotoleranța și să trateze boala. Datele noastre demonstrează că unele produse IVIG conțin o cantitate semnificativă de anticorpi AFab2 și AH. Aceste rezultate deschid perspectiva terapiei substitutive a unor boli în care acești anticorpi imunosupresivi joacă un rol patogenic.

Keywords: IVIG, immunotolerance, anti-F(ab')2

Introduction

Intravenous immunoglobulins (IVIGs) are pooled immunoglobulins (IGs) products obtained by purification from plasma of healthy donors containing high concentrations of IgG antibodies, low concentrations of IgA and IgM, cytokines and cytokine antagonists [6, 21]. All these active substances may contribute to therapeutic effects. The half-life time of IgG after intravenous infusion is approximately three to four weeks [1, 3].

IVIGs are used as replacement therapy after blood loss, in sepsis, immunodeficiency syndromes (primary or secondary) or as immunomodulators in auto-immune diseases (e.g. idiopathic thrombocytopenic purpura, Kawasaki disease) or graft recipients [7]. Studies also analysed the efficiency of IVIG therapy in other pathologic conditions such as Guillain-Barres syndrome, myasthenia gravis, foetal alloimune neonatal thrombocytopenia, foetal haemolytic disease, dermatological diseases or spontaneous abortion [7].

Many studies have contributed to the understanding of their mechanism of action and showed that IVIGs exert their effects on T-cells, cytokines, B-cells, complement system and Fc-receptors [6]. However, the mechanism of action of IVIGs has not been fully clarified. Therefore, research concerning the biological activity of different components of IVIGs could contribute to better understand the mechanism of action and pave the way for an extended clinical use of IVIG.

Because IVIGs are obtained from purified immunoglobulins collected from an increased number of donors, they contain a huge variety of IgG with a large array of specificities. Previous studies performed in our, as well as other laboratories, documented that AFab2 are present in the immune repertoire of both healthy persons, persons with autoimmune diseases, and graft recipients [20, 22, 25, 29]. That is why it is expected that AFab2 are part of IVIG products.

Our previous results showed that AFab2 abs exert an immunosuppressive activity *in vitro* and *in vivo*. AFab2 abs suppress anti-erythrocyte autoantibody producing B-cells *in vitro* [26]. Research conducted *in vivo* showed that AFab2 increase after immunization. We speculated that their physiological role is to limit the extent of a successful immune response [24, 26]. Studies carried out on sera from patients with cold agglutinins and lupus erythematosus bring evidence that a higher AFab2 concentration correlates with a lower pathogenic autoantibody titer and *vice versa*, suggesting a control of the autoimmune B-cell clones by the AFab2 [22, 29]. The research group around the Collaborative Transplant Study (CTS) in Heidelberg showed that allograft recipients with a high pre-transplant AFab2 titer had a lower rejection rate compared with those with lower AFab2 titers [17, 18, 19]. Moreover, first trimester sera of pregnant women with birth at term showed higher AFab2 concentration compared with non-pregnant women and pregnant women with spontaneous abortion or spontaneous preterm birth [13]. All these results argue for an immune-suppressive mechanism exerted by AFab2 [24]. Should low AFab2 be pathogenically involved in all these conditions, a therapeutic increase of the Ab titer by administration IGIG may ameliorate the disease.

The aim of our study was to research if IVIGs contain AFab2 and AH antibodies and to analyse how AFab2 and AH could contribute to the immunomodulatory effects of IVIGs.

Materials and Methods

IVIG products

IgG, AFab2 and AH antibodies concentration were measured in five different IVIG commercial products [Endobulin® (Immuno), Gammonativ® (Pharmacia GmbH), Gammagard® (Baxter), Sandoglobulin® (Sandoz), and Intraglobin® (Biotest PharmaGmbH)].

Measurement of IgG concentration in IVIG products

The concentration of IgG was measured in IVIG products by nephelometry using Minineph (The Binding Site Group Ltd, Birmingham, UK).

Detection of IgG-anti-F(ab')2 antibody concentration in IVIG products

Microtiter plates (x96) (Nunc - Maxisorp flatt bottom 96 well plate) were coated with 1 μg/well of human $F(ab')_{2x}$ fragments. Remaining active groups were blocked with phosphate-buffered saline (PBS) + 1% gelatine. Prediluted IVIG's (12 mg/mL) were further diluted 1:32/1:64/1:128/1:256 in phosphate buffer saline (PBS) and applied to precoated plates (50 μL/well) in triplicates. A reference serum with known AFab2 activity was used as positive and PBS as negative control. After incubation, 50 μL of alkaline phosphatase-conjugated goat anti-human IgG(Fc) antibody (Jackson Immunoresearch) were added. Each step was followed by extensive washing with PBS + 0.05% Tween. One hundred fifty μL substrate (250 μg p-nitrophenyl phosphate disodium/well) (Sigma Chemical CO, St Louis, MO) was added and the extinction was measured at 405 nm. The test was stopped at an extinction of 1500 in the positive control.

Detection of antipeptide (anti-Hinge) antibody in IVIG

A double-chain IgGl Hinge peptide *(Mr* = 2503) comprising the core and lower hinge region of IgG1 (225-237/225'-237') was used in this study (Figure 1).

H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-Glu-Leu-Leu-Gly-Gly-OH
| |
H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-Glu-Leu-Leu-Gly-Gly-OH

Figure 1.
Structure of the synthetic long Hinge peptide related to the Hinge region of IgG1

The synthesis of the peptide was performed by assembling two suitably protected fragments via the classical methods of synthesis in solution as described previously [8, 10, 23]. Nuclear magnetic resonance analyses and circular dichroism spectroscopy [8, 10, 23] showed that the hinge peptide has a defined conformation. The three dimensional structure of its cyclic portion is almost identical tthe core hinge of the intact IgG1 molecule, comprising a fold of two poly(Pro)-II helices [8, 10]. Microtiter plates were coated with 50 μL/well of hinge peptide (7 μM). Remaining active groups were blocked with PBS + 5% gelatine. Prediluted IVIGs (12mg/mL) were further diluted 1:16/1:32/1:64/1:128 in PBS and applied (50 μL/well) to precoated plates in triplicates. A reference serum with known anti-Hinge activity was used as a positive and PBS as a negative control. In the next step, 50 μL of alkaline phosphatase-conjugated goat anti-human IgG(Fc) (Jackson Immunoresearch) was added. Each step was followed by extensive washing with PBS + 0.05% Tween. Substrate (250 μg p-nitrophenyl phosphate disodium/well) (Sigma Chemical Co., St.

Louis, MO) was added and the extinction was measured at 405 nm. The test was stopped at an extinction of 800 in the positive control.

Results and Discussion

IgG antibodies concentration in IVIG products

Commercial IVIG preparations showed different IgG titers. The IVIGs were pre-diluted (for subsequent testing) to an IgG concentration of 12 mg/mL, equivalent to an IgG concentration in plasma of healthy pro-bands (Table I).

Table I

Concentration of IgG antibodies in IVIG products

No.	IVIG Product	Concentration of IgG in IVIG products (mg/mL)	Pre-dilution	End-concentration of pre-diluted IVIGs (mg/mL*)
1	Gammonativ®	51.1	1:3.425	12
2	Endobulin®	57.7	1:4.808	12
3	Gammagard®	41	1:3.416	12
4	Sandoglobulin®	30.3	1:2.525	12
5	Intraglobin®	45.8	1:3.816	12

* 12 mg/mL - Normal value of IgG concentration in plasma of healthy subjects

Pre-diluted IVIGs were diluted for subsequent testing according to Figure 2 and Figure 3.

The IgG-anti-F(ab')2 antibody titer in IVIG products

Various IVig preparations showed different AFab2 titers at the same IgG concentration. The highest AFab2 concentration was found in Endobulin followed by Gammonativ®, Gammagard®, Sandoglobulin® and Intraglobin® (Figure 2).

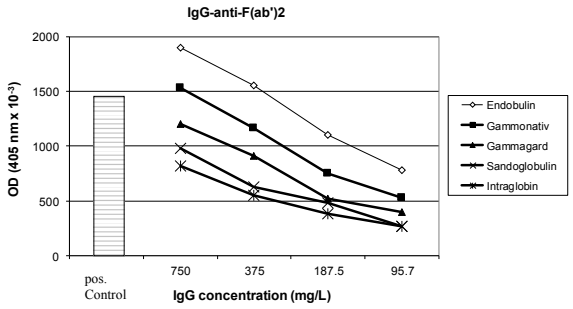

Figure 2.

IgG-anti-F(ab')2 autoantibodies concentration in IVIG products

The IgG-anti-F(ab')2 antibody titer (abscissa) was measured in IVIG products diluted at different IgG concentrations (ordinate). Different IVIG products showed different IgG-anti-F(ab')2 titers.

The IgG-anti-Hinge antibody (AH) titer in tested IVIG products

One particular antibody within the AFab2 family was the IgG-anti-Hinge antibody. IVIG products showed different AH levels at the same IgG concentration. The highest AH concentration was found in Endobulin® followed by Gammonativ®, Gammagard®, Sandoglobulin® and Intraglobin® thus paralleling the AFab2 titer (Figure 3).

Our results showed for the first time that IVIGs contain both AFab2 and AH. This observation supports the hypothesis that some of the immunomodulatory effects of IVIG products are caused by the immunosuppressive features of the two antibodies.

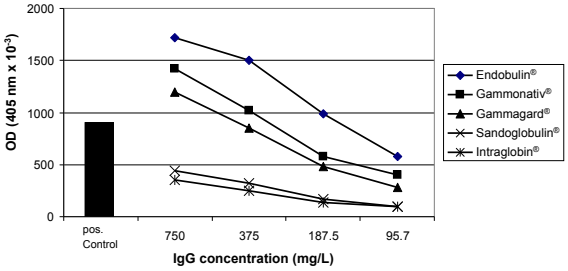

Figure 3.

IgG-anti-Hinge autoantibodies concentration in IVIG products

The IgG-anti-hinge antibody titer (abscissa) was measured in IVIG products diluted at different IgG concentration (ordinate). Different IVIG products showed different IgG-anti-Hinge titers.

Many other mechanisms were suggested to explain the immunomodulatory effect of IVIGs in patients with autoimmune diseases. One of them implies that the immunosuppressive effect of IVIGs is caused by anti-idiotypic antibodies [28]. It is well known that IVIGs contain a high concentration of anti-idiotypic antibodies. According to this mechanism the anti-idiotypic antibodies block the binding of autoantibodies to autoantigens, weakening the autoimmune reactions [6, 28]. Nagelkerke proposed another mechanism of IVIGs action by stating that the delivery of IVIGs increases the IgG concentration and those block competitively the binding to Fc-receptors in competition with autoimmune IgG's [12]. Other studies argue for the presence in IVIGs of antibodies against complement components, or stimulatory interleukins. Nowadays all these mechanisms are considered to be potentially

responsible for the immunomodulatory effect of IVIG.

Our previous studies showed that AFab2 antibodies are part of the physiological and pathological mechanisms and argue for an immunosuppressive role of AFab2 [24]. Since IVIG contain AFab2 in high concentrations it is expected that some of the effects of IVIG may be caused by these antibodies.

This immunosuppressive effect might be based on a mechanism proposed by Chan, Sinclair and others who showed that the crosslinking of the Fc-receptor with the antigen-receptor (membrane immunoglobulin) induces the suppression of the B cell activity [2, 4, 15, 16, 24]. The cross-linking of the two receptors is possible if AFab2 bind with their antigen binding site to the membrane immunoglobulin of B cells and with the Fc region to the Fc-receptor gamma II [24]. Such cross-linking prevents the influx of Ca^{2+} into the cell by closing the plasma Ca^{2+} channels. Without intracellular Ca^{2+}, the lymphocytes cannot be activated [5, 11]. Because B cells are antigen-presenting cells and contribute to the regulation of T cells by various cytokine, AFab2 that suppress B-cells would indirectly influence T cell activity [14].

Studies carried out by our research group showed that the inhibitory effect of AFab2 is dose-dependent. An optimal concentration of the antibody is necessary to induce B-cell suppression. This concentration depends on the antibody´s affinity towards the membrane immunoglobulin and Fc receptor [26]. Apparently, the AFab2 antibody binds with highest affinity to immunoglobulin-antigen complexes. Consequently, not all B-cells would be suppressed, but rather those whose membrane immunoglobulin is occupied by an antigen [2, 24]. This applies to autoantigens, alloantigens in pregnant women and graft recipients. In fact, patients with autoimmune diseases, spontaneous abortion, *in vitro* fertilization (IVF) failure and graft recipients are potential candidates for an immunomodulatory therapy with IVIG (AFab2 and AH).

Our previous studies showed that AFab2 are a family of Ab within which some are not anti-idiotypic Ab, but rather bind to epitopes located in the constant region of the IgG molecule [23]. One such member is the anti-Hinge antibody. Interestingly, AH titers in the tested IVIG preparations paralleled those of AFab2 antibodies. This lets us speculate, that AH are one immunoregulatory component of IVIGs. Moreover, the fact that the AFab2 described by us recognize constant IgG-region epitopes provides an explanation why these non-idiotypic AFab2 exert an immunosuppressive effect in various types of pathologies, such as autoimmune diseases [22, 29], allograft rejection [19, 20], spontaneous abortions and preterm birth [13].

The results of studies about the efficiency of IVIGs in patients with certain autoimmune diseases, IVF failure or recurrent miscarriage are controversial [9, 27]. We suspect that the difference between the efficiency of IVIGs therapy in these patients is caused by an insufficient stratification of patients or by an inadequate selection of commercial IVIGs preparations according to AFab2 or AH titers. Of course, this hypothesis should be proved in large prospective studies.

Conclusions

Our results show that IVIGs contain AFab2 and AH Ab in significant concentrations. They pave the way for studies evaluating the effect of IVIGs therapy according to the AFab2 and AH concentration in both the serum of patients and the therapeutic preparations.

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