Anti-Red Blood Cell Antibodies, Free Light Chains, and Antiphospholipid Antibodies in Intravenous Immunoglobulin Preparations

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ABSTRACT: **Background:** Anti-red blood cell antibodies, free light chains (FLC) and prothrombotic proteins (PTP) may co-elute with intact immunoglobulin (IgG), and may be the cause of adverse reactions to intravenous immunoglobulin preparations (IVIG).
Objectives: To investigate the presence of residual amounts of these components in IVIG and their effects on ABO blood group agglutination.
Methods: Iso-agglutinin anti-A and anti-B activity was determined with a direct hemagglutination assay of red blood cell (RBC) suspensions from 1% of 46 blood donors together with the serial dilutions of five IVIG (IV1, IV2, IV3, IV4, IV5). Anti-A1 monoclonal antibody was used to confirm reactivity with the A1-reference RBC. The selected IVIG were diluted to a final concentration of 25 mg/ml in 0.15 M NaCl and 0.01 M phosphate-buffered saline, pH 7.4, with or without a further twofold dilution in a low ionic strength solution.
Results: A variation up to fivefold in the titer strength of anti-A/B activity was observed between the IVIG preparations. A2-type RBC required higher IVIG inputs when tested in 0.15 M NaCl. The differences in FLC kappa and lambda concentrations were as high as › 400 mg/L among the various IVIG. Only IV1 had a significantly high level of antiphospholipid IgG antibodies (18 U/ml). We demonstrated the presence of anti-RBC antibodies, FLC and PTP in IVIG preparations.
Conclusions: Our findings provide clear evidence that IVIG may harbor pathophysiological substrates with a potential risk for adverse effects such as iatrogenic hemolysis, FLC-associated disorders, and thromboembolism.

IMAJ 2013; 15: 617–621

KEY WORDS: intravenous immunoglobulin (IVIG), red blood cells, hemagglutination, free light chains (FLC), phospholipids

The efficacy of each active pharmaceutical ingredient in polyclonal and polyspecific intravenous immunoglobulin preparations, as well as the extent of its side effects, varies among patients. The incidence of immune hemolysis following IVIG administration has increased over the years [1]. Anti-A/B IgG antibodies from the blood type ABO are sometimes found in IVIG and are able to activate complement. The interactions between anti-A/B IgG and patients with A, AB or B blood types have garnered increasing attention recently after being virtually ignored for many years [2]. The antigens in the ABH, Lewis, P and I blood group systems are synthesized through interrelated pathways, indicating that anti-A/B antibodies may obscure other blood-type specificities that would be targeted by the anti-RBC antibodies in IVIG, including RhD. The hemolytic capacity of IVIG in recipients is independent of ABO type, although A-type patients appear to be at greater risk [3]. The increased interest in post-IVIG hemolysis is due in part to an increase in the concentration of IVIG administered to patients, from 5% to 12%. Some agencies have recommended a slower transfusion rate of 0.5–1.0 ml/kg body weight/height for the initial treatments [2]. Currently, there is no formal consensus defining the gold standard laboratory technique for detecting anti-A/B antibodies in IVIG products. Sensitive techniques, such as FACS or ABO-ELISA, may be more appropriate than the anti-globulin tests or direct agglutination tests that are currently used [4,5].

Differential reactivity of intact 7S IgG or dimers is possible, and although free Fc fragments can attenuate autoimmunity-initiated inflammation [6], they can be used as active pharmaceutical ingredients in their purified form [7]. Free light chains have never been considered active pharmaceutical ingredients. They trigger mast cells to interact with dendritic cells at the sites of inflammation [8]. Because amyloid precursors are mediators of organ dysfunction and the ensuing deposition of disease [9], the presence of free light chains in IVIG is of concern.

The risk of thromboembolic complications caused by IVIG therapy is not negligible [10]. Pro-coagulatory contaminants in IVIG are usually removed; however, the spectrum of deter-

IVIG = intravenous immunoglobulin
igG = immunoglobulin G
ELISA = enzyme-linked immunosorbent assay

minants may also include anti-A/B antibodies which target the endothelial sites of histoblood group ABH antigens, followed by immune complex formation that is known to favor thrombosis. To date, hyperviscosity conferred by infusion of the immunoglobulin concentrates is thought to be the main reason for the occurrence of venous and arterial thromboembolism [11]. However, immunogenic thromboembolism is one of the features of antiphospholipid syndrome. Theoretically, IVIG could contain thrombogenic immunoglobulins, which would increase the risk of thromboembolism in IVIG recipients.

To further characterize potentially harmful compounds in IVIG, we analyzed five different preparations of IVIG for the presence of anti-ABO-type antibodies, FLC, and prothrombotic proteins, such as antiphospholipid antibodies.

MATERIALS AND METHODS

The five IgG preparations selected for this study are approved for market access by the Swiss Agency for Therapeutic Products, Swissmedic. All preparations were used prior to their expiration dates.

HEMAGGLUTINATION ASSAY

Isoagglutinin anti-A and anti-B activity was determined with a direct hemagglutination assay. In this assay, 1% red blood cell suspensions were used with serial dilutions of the IVIG preparations. RBC from blood types A1, A2, A1B, A2B, B and O were obtained from regular blood donors at the Swiss Transfusion Service. ABO typing was performed according to national standards at this location. For type A1, B and O, 13 donors of each type served as the basis for the hemagglutination assay. For type A2 we used four donors, for type A1B two donors, and for type A2B one donor. The anti-A1 monoclonal antibody was kindly provided by G. Halverson (New York Blood Center and LFKRI, Laboratory of Immunochemistry, New York). This antibody was used to confirm the reactivity of the A1-reference RBC from the Swiss provider. Different dilutions of IVIG were used so that each of the five IVIG preparations was normalized to 25 mg/ml of IgG. As diluents, we used 0.15 M NaCl and 0.01 M phosphate-buffered saline, pH 7.4. Both of these buffers were diluted twofold with a low ionic strength solution (LISS Diluent™, BioRad®, Cressier sur Morat, Switzerland), and the pH was adjusted appropriately. The serial dilutions of the IVIG preparations were begun at concentrations of either 25 or 50 mg/ml. The dilution factor, expressed as the conventional agglutination titer (reciprocal of serial dilution), is indicated in Table 1 and Figures 1 and 2, along with the mg/ml protein input. Prior to the reading of the streaking patterns by two independent observers, the plates were incubated for 1 hour at 37°C and then overnight at 4°C. It is important to note that high protein concentration values necessary for agglutination to occur indicated a lower anti-RBC content. The gel card assay was performed using standard procedures [12].

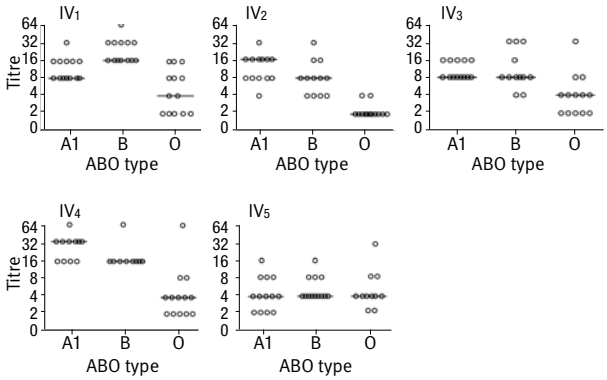
FLC = free light chains
RBC = red blood cells

Table 1. Dilution titers observed with the Diamed microcolumn gel assay using the NaCl cards (BioRad®, Cressier, Morat, Switzerland)

	A1	B	O
IV1	16*	8	4
IV2	8	4	1
IV3	16	8	2
IV4	32	16	2
IV5	4	2	2

*Reciprocal of dilutions

Figure 1. The different concentrations of IVIG preparations required to agglutinate A1-, B- and O-type RBC. Five IV immunoglobulin preparations – IV1, IV2, IV3, IV4 and IV5 – were assayed for RBC agglutinating capacity in PBS by a direct hemagglutination assay. Upper panel: reciprocal of serial dilutions are plotted on the ordinate; lower panel: the inverse representation of the protein concentrations needed to produce hemagglutination in all donors are plotted on the ordinate. Note that the higher the IVIG concentration needed, the lower its anti-A/B content



NEPHELOMETRY

The FLC were quantified using nephelometry (BN Prospec, Siemens Healthcare Diagnostics, Switzerland) with polyclonal antibodies. This assay was used to identify the hidden light chain epitopes of free κ and λ chains that were not cross-reactive to the κ and λ epitopes on intact immunoglobulins (FreelightTM Binding Site GmbH, UK). The total protein concentration was assayed with the Micro BCA Protein Assay Kit using the bicinchoninic acid formulation for colorimetry (Thermo Scientific, Rockford, IL, USA). Albumin (20%) (CSL Behring, USA) was used as a background signal control. Twelve normal human serum samples and 8 follow-up clinical samples from male and female patients, aged 23–78 years, with known elevations of FLCs were used to study the ratio between the light chains

contained in total IgG and FLC. The 95% reference interval for FLC κ is 3.3–19.4, and for FLC λ 5.7–26.3 mg. This comparison demonstrated both the sensitivity (0.3 mg/L) and reproducibility, as outlined by the reagent manufacturer (www.freelite.co.uk).

ELISA

The presence of antiphospholipid antibodies was investigated using commercial kits. Initially, the different IVIG preparations were tested with antiphospholipid antibody screening tests for IgG and IgM (Antiphospholipid Screen IgG, Anti-phospholipid Screen IgM, Orgentec Diagnostika®, Mainz, Germany). Positive screening results were then further characterized with monospecific tests for anti-β2-glycoprotein I, anticardiolipin, anti-phosphatidylserine, anti-phosphatidylinositol, and anti-phosphatidic acid (Orgentec Diagnostika®, Mainz, Germany). All tests were performed according to the manufacturer's instructions.

STATISTICAL PROCEDURES

Mean, median and comparisons were performed using the Prism GraphPad™ Software, which was also used for scientific graphing.

RESULTS

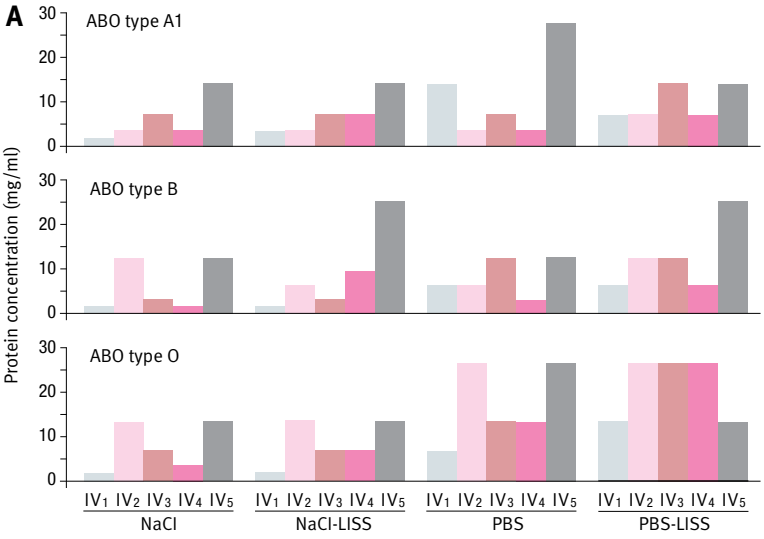
ANTI-RBC ANTIBODIES

The IVIG preparations were first screened for anti-RBC hemagglutinins. The dilution factors and protein concentrations (mg/ml) that resulted in agglutination in the direct hemagglutination assays with type A1-, B-, and O-type RBCs ranged from 2 to 64 (reciprocal of dilution), and from 25 mg/ml (low titer) to 1.56 mg/ml (high titer) [Figure 1]. With the IV2 and IV5 preparations, more protein was necessary to obtain hemagglutination than with the other preparations tested. IV2 and IV5 contained the lowest concentration of anti-A isoantibodies of all the IVIG preparations. It should be stressed that type O RBC were also a target for anti-RBC antibodies as the type A and B RBC. The agglutination capacity of the five different IVIG preparations tested against the five different ABO types depended on the test conditions, to some extent. As expected, LISS enhanced agglutination, whereas PBS and PBS-LISS caused weaker patterns of agglutination than the NaCl condition [Figure 2A]. The results obtained with the classical hemagglutination system were determined with gel card technology [Table 1] and demonstrated consistent results.

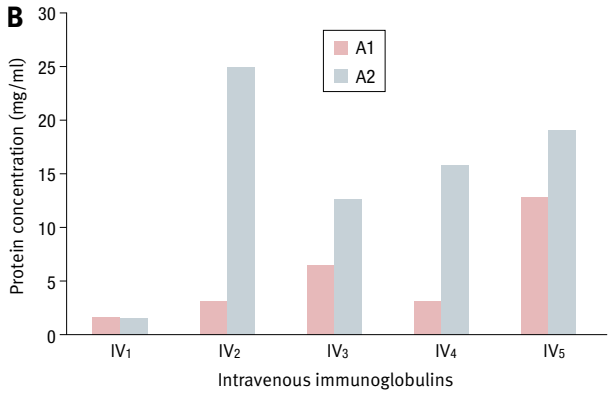
Since A1-type expresses higher A-epitope density on RBC than A2, we further investigated the influence of A1/A2 subtypes and hemagglutinin avidity, and pH and molarity of the buffers on the agglutination titers. Most preparations exhibited stronger titers when tested with A1 cells rather than A2 cells [Figure 2B].

LISS = low ionic strength solution
PBS = phosphate buffered saline

Figure 2. [A] Influence of the incubation buffer on hemagglutination. Incubation buffers tested: 0.15 M NaCl, PBS and LISS (abscissa) are compared with regard to their influence on the protein concentrations needed to produce hemagglutination of five different IVIG preparations (see Table 1). Five blood donors of type A1, B and O were used for these experiments, and median values of five duplicate experiments. Note the variation in results independent of the buffers, with NaCl-LISS favoring agglutination



[B] Influence of A subtype on hemagglutination. Five blood donors with the indicated A subtype donated red blood cells for these experiments done in NaCl milieu. The A1 RBC require less protein input from IVIG preparations than the A2 RBC. The bars represent the median values of five different experiments, performed in duplicate and read by two independent readers



FREE LIGHT CHAINS IN IVIG

The IVIG preparations were also screened for the presence of FLC. FLC concentrations as high as 90 mg/L and 55 mg/L were obtained for free κ-chains and free λ-chains, respectively [Table 2]. When expressed as a percentage of the total IgG contained in the preparations, free κ-chains and λ-chains were valued at 0.36% and 0.20%, respectively. In the 20% albumin control group, no free κ-chains or λ-chains were detected (< 0.001%).

Table 2. Concentrations of free light chains and antiphospholipid screening in different IVIG preparations

	FLC κ* (mg/L)	FLC λ (mg/L)	Antiphospholipid screening IgM**	Antiphospholipid screening IgG**
IV1	156.0 ± 6.8	218.8 ± 8.7	2.7	18.0
IV2	21.0 ± 0.4	22.2 ± 0.5	2.1	2.6
IV3	57.7 ± 1.3	57.4 ± 1.7	2.1	4.3
IV4	77.2 ± 2.3	51.7 ± 1.7	2.0 + 1.8	3.2 + 4.1
IV5	423.6 ± 35.2	230.4 ± 18.0	2.1	4.3

*Values are mean and standard errors of three independent nephelometric measurements of free light chains kappa (FLC κ) and free light chains lamda (FLC λ)
**Mean ± SE of three FLC measurements. Cutoff for antiphospholipid screening was 10 U/ml

PROTHROMBOTIC ANTIBODIES

Screening of the IVIG preparations for antiphospholipid antibodies revealed that none of these products had IgM isotype antiphospholipid antibodies [Table 2]. However, one of the preparations, IV1, had a positive screening result for antiphospholipid antibodies, with a total of 18 U/ml. Further investigation identified anticardiolipin, anti-β2-cardiolipin, anti-phophatidylinositol, anti-phosphatidylserine and anti-phosphatidic acid antibodies in the IV1 preparation, indicating the presence of the entire spectrum of antiphospholipid IgG antibodies in this product.

DISCUSSION

Each of the five tested IVIG preparations had anti-RBC antibodies as determined by hemagglutination assays. RBC samples obtained from different donors of the same ABO type showed distinct binding specificities to anti-A, anti-B and anti-RBC antibodies from the same IVIG preparation. IVIG preparations are widely known to contain anti-A/B and anti-RBC antibodies beyond the ABO-type specificity spectrum [13]. Most reports base these findings on observing patients with hemolysis; however, the century-old method of hemagglutination is often used without validation under GLP directives in standard guidelines, as in CLSI or Merck. Therefore, efforts are currently underway to fill this gap [5]. The anti-A/B antibodies were most pronounced against A1-, A2- and O-type RBC, in that order; however, within each type of preparation the concentration of each varied considerably. While we did not perform antiglobulin enhancement experiments because of the neutralization of antiglobulin reagent by excess IgG [14], or from lectin or soluble ABH antigen inhibition experiments, it has been postulated that the anti-O-type reactions may target carbohydrate-related RBC antigens, such as Lewis, P and I. Therefore, the latter three blood types will be less active in the clinical setting, with A- and B-type patients being more prone to hemolysis reactions than O-type individuals. The variable concentrations of anti-RBC in the preparations used in this study may be due to the inconsistent quantities of anti-RBC during the formation of the plasma pools for fractionation. The concentrations may also vary because of putative, undeclared absorption prior to market release, or the exclusion of O-type donors from the plasma pool.

Our observations may be a result of the following: a) disparity in the glycosylation patterns secondary to the effects of the glycosyltransferases that synthesize ABH-antigens; b) inconsistent expression of epitopes and/or epitope density, both of which are known to vary, even among RBC of the same A subtype and across polygenic anti-A traits [15]; and c) hidden blood group specificities such as Rhesus, Lewis, Duffy, and Kidd, which may account for the agglutination of O-type cells observed here and in previous studies. The RBC of identical ABO-type donors required slightly different IgG concentrations for agglutination depending on the reaction buffer. It remains unclear whether NaCl-LISS favored agglutination over the phosphate buffer in LISS. The latter buffer system likely caused a weaker antigen-antibody interaction because of the reduced concentration of H^+ ions. It is difficult to compare the anti-A/B content of IVIG across laboratories, regardless of whether the laboratories adhere to GLP quality systems. However, efforts are underway to standardize laboratory testing [5]. ELISA and flow cytometric analyses have also been used to quantify anti-A/B antibodies [16]. Presently, the specificity of clinically relevant, harmful components in IVIG preparations is best assessed using natural antigens of the human type-A1 RBC.

For physicians and patients alike, it is important to quantify the amount of anti-A/B antibodies in IVIG preparations for two reasons: a) anti-A/B antibodies are linked to the risk of hemolytic episodes in the recipient [2], particularly if he or she is type-A1; and b) the amount of anti-A antibody in IVIG may be critical when IVIG is prescribed in ABO-incompatible organ transplantation. In the latter condition, a low level of anti-A antibody in the IVIG provides better conditioning of the ABO-incompatible solid organs [17], which are known to carry significant amounts of the ABH histoblood group antigens [18].

Our study is the first to document the presence of FLC in immunoglobulin preparations from different manufacturers. Although a variety of methods were used by the manufacturers to transform their products into the specific polyclonal and polyspecific IVIG, the five preparations studied for FLC content were prepared by similar processes of fractionation. The preparations were used prior to their expiration dates, but one cannot exclude the effect of storage conditions on FLC values.

The API-like presence of FLCs in immunoglobulin preparations is of concern, given the potential for harmful FLC-induced effects, such as renal disease [19]. Kidney damage following the infusion of IVIG has been reported [20]. Initially, sucrose, a compound still in use to stabilize some IVIG products, was deemed responsible for the majority of kidney

GLP = good laboratory practice

damage occurring in patients. The remaining kidney damage could also be triggered by a sharp but transient increase in FLC contained in IVIG. High dose IVIG treatments, such as 2 g/kg body weight for 3 days, may increase the recipient's FLC concentration above 10 mg/ml, possibly altering the normal kappa/lambda ratios [21].

Our investigation indicates that IVIG may also contain antiphospholipid antibodies. These antibodies occur in antiphospholipid syndrome. Theoretically, it may be possible that IVIG administration also confers thrombogenic antibodies. Venous and arterial thromboembolisms have been reported as adverse events of IVIG administration [22]. As a mechanism, hyperviscosity has been postulated, and rapid infusion rates are thought to increase the risk of thromboembolism [11]. Our investigations offer another mechanism of transfused hypercoagulability due to thrombogenic antibodies. The theory of an "iatrogenic antiphospholipid syndrome" caused by IVIG would be consistent with the fact that antiphospholipid syndrome causes both arterial and venous thromboembolisms [23].

This study is limited by the fact that we analyzed only one lot of each immunoglobulin preparation and that we could not identify the specificities of the anti-RBC antibodies. It may be possible that the substances investigated differ not only among the different preparations but also among different lots of the same preparation. Furthermore, we provided evidence of the presence only of potentially harmful substances in IVIG. We did not investigate whether these substances effectively cause harm to patients. However, the literature provides accruing evidence that hemolysis [24] and thromboembolism occur in conjunction with IVIG administration, and organ damage can occur in association with immunoglobulin light chains [25]. Taken together, we do not believe that these limitations invalidate our findings.

CONCLUSIONS

Studies on non-nominal, side effect-triggering components in IVIG preparations are conducted in the post-marketing surveillance setting or once these preparations have caused adverse effects. Here, we prospectively address this topic by listing major analytical sources of variation. We also detected unanticipated components in IVIG that may be considered inducers of adverse events.

Acknowledgments

The study was financed with an in-house research grant at Labormedizinisches Zentrum to Dr. Risch, Bern, Switzerland

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