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Review

Immunochemical-based assays in the bioanalysis of immunoglobulins

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Abstract

Intravenous immunoglobulin (IVIG) preparations consist of IgG derived from a pool of human plasma of healthy individuals and have been used as routine treatment of patients with primary and secondary immunodeficiencies, autoimmune, and/or inflammatory diseases. Emergence of new infectious agents and development of antibiotic resistance in many bacteria have posed serious problems in the treatment of infections. Since IVIGs contain natural antibodies that occur in the healthy population, their administration to immunocompromised hosts either as a prophylactic agent or as complementary treatment to the usual antimicrobial treatment have been studied. Contradictory results obtained by several clinical studies in respect to the clinical efficacy of IVIGs have in part been ascribed to the poor characterization of IVIG preparations in terms of their specific antibody content against the various pathogenic microorganisms. Immunoassays constitute a promising tool for bioanalysis of IVIGs thanks to the high sensitivity, repeatability and ease of implementation. Ensuring high selectivity, enzyme immunoassays have been used for determination of the levels of pathogen-specific antibodies in IVIG preparations. In this review, the application of immunoassays monitoring such specific antibodies in IVIGs and the relationship of estimated titers with their in vitro opsonic activity are summarized. The relationship of the content of specific antibodies in IVIGs and their functional efficacy with the outcome of clinical studies including patients with primary immunodeficiencies and premature neonates treated with IVIGs is also discussed.

Keywords: Enzyme immunoassay; Intravenous immunoglobulin; Specific antibodies; Primary immunodeficiency; Neonatal infection; Bacterial antigens

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1. Intravenous immunoglobulin preparations

The advent of intravenous immunoglobulin (IVIG) preparations signaled a new era in clinical immunology since their infusion is a safe, effective and painless way of administering protective antibodies to immunodeficient or immunocompromised patients [1]. They consist of IgG antibodies derived from plasma of a very large number of healthy donors and therefore IVIGs contain all the antibodies that normally occur in the healthy population. They are currently used for substitution therapy of primary and secondary immunodeficiencies, as treatment of idiopathic thrombocytopenic purpura (ITP), Kawasaki disease and certain other autoimmune and inflammatory diseases, and as prophylactic agent in patients at high risk to develop severe infections [2–4]. Retrospective studies on the outcome of prolonged treatment of primary antibody deficiencies (X-linked agammaglobulinaemia and hypogammaglobulinaemia) have demonstrated the effectiveness of IVIG in preventing severe infections [5,6]. In general, children who receive IVIG replacement therapy grow normally and exhibit infection rates similar to those of healthy children. IVIG effectiveness, however, in prevention/treatment of infection in preterm neonates remained controversial despite the great number of clinical trials [7,8]. Preterm neonates are not only quantitatively deficient in IgG antibodies due to incomplete transfer of maternal IgG, but also qualitatively deficient since placental transfer of IgG2 molecules (antibodies mainly against capsular polysaccharide antigens) is less favorable. Furthermore, neonates are deficient in antibodies that the mother does not supply due to lack of previous exposure(s).

IVIG exerts anti-infective function through a variety of mechanisms. The most obvious mechanism is passive administration of antibodies specific to bacterial surface antigens, which facilitate inhibition of microbial attachment, complement activation and opsonization. IVIGs also contain antibodies to bacterial toxins and superantigens that neutralize their action. Furthermore, some of the immunomodulatory effects leading to down-regulation of cytokine secretion, endothelial cell activation and lymphocyte function, which are hypothesized to improve autoimmune disease, may also prevent infection or minimize its consequences.

According to World Health Organization guidelines, the content of antibodies to two, at least, microorganisms or toxins and two viruses must be determined in every IVIG lot [9]. Given the vital role of pathogen-specific antibodies, confidence in the capability of an IVIG preparation to accomplish the desired anti-infective end result would be enhanced if titers of antibodies to a greater range of pathogens were available. However, the lack of standard preparations for most antibodies hinders their exact quantitative measurement. Currently, pathogen-specific antibodies in IVIGs are measured by haemagglutination inhibition tests, neutralization tests, complement fixation reactions, opsonic assays and immunoassays, such as radioimmunoassay, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence. A representative case in which the presence of specific antibodies in an IVIG preparation against slime-producing *Staphylococcus epidermidis* is shown using immunofluorescence is given in Fig. 1. Immunoassays, in general, exhibit high sensitivity, specificity and reproducibility, but little is known about the clinical relevance of the estimated titers.

In this article, applications of immunoassays in the determination of antibodies specific to bacterial isolates and discrete antigenic components, e.g. toxins and superantigens, are reviewed and the relationship of the estimated titers with the in

Fig. 1. Immunofluorescence profile showing the presence of specific antibodies against slime-producing *S. epidermidis* in an IVIG preparation (Sandoglobulin®). Immobilized cells were incubated with Sandoglobulin® diluted at 1:80 in PBS and visualization was performed by further treatment with goat anti-human IgG conjugated with fluorescein.

Fig. 2. Documentation of the opsonic activity of Sandoglobulin® against slime-producing *S. epidermidis* using Gram (A) and methylene blue (B) staining. Bacteria were incubated with human polymorphonuclear cells in a ratio 1:1 in the presence of Sandoglobulin® and in the absence of complement for 90 min.

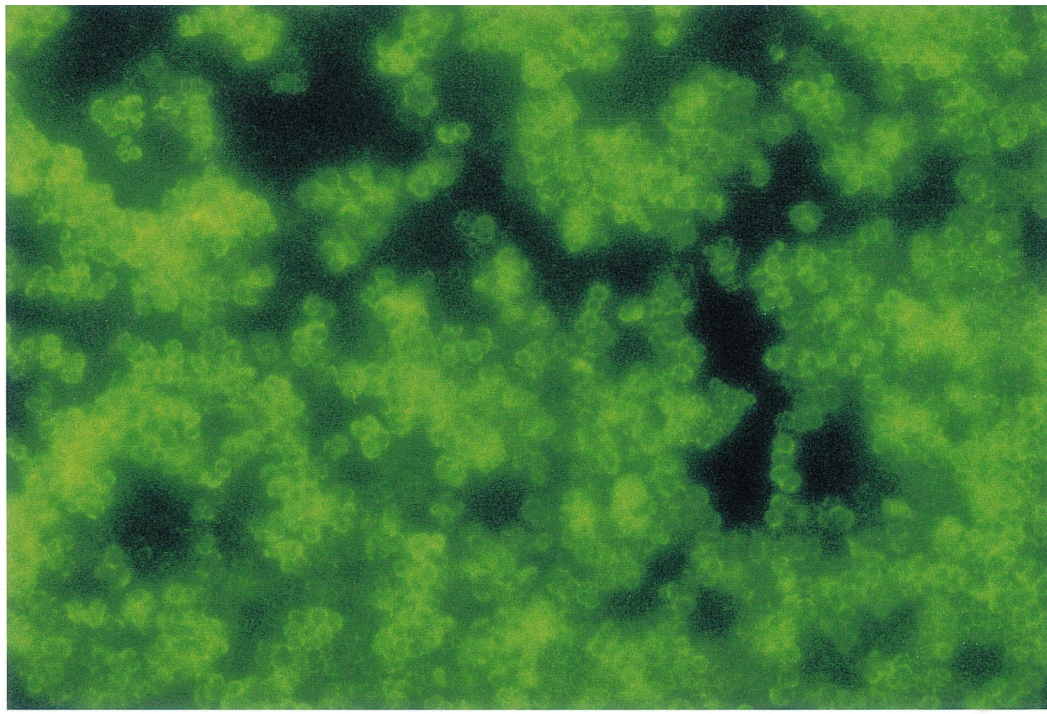

Fig. 1

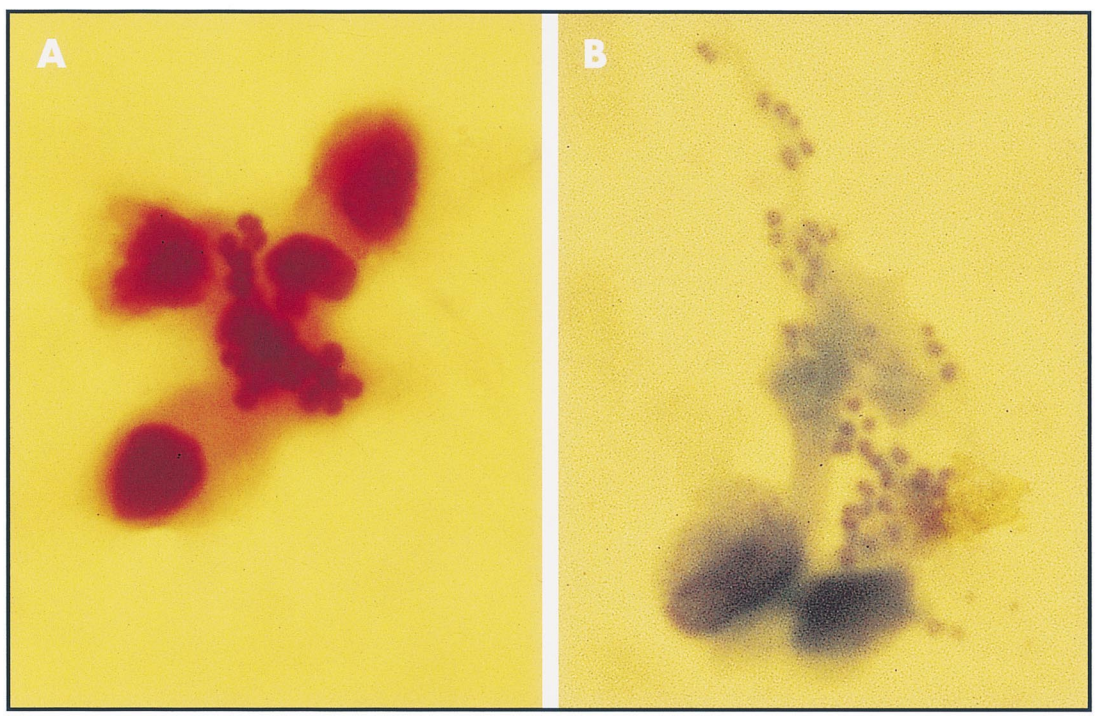

Fig. 2

vitro opsonic activity or results of in vivo clinical studies is presented.

2. Immunoassays for monitoring antibodies against bacterial isolates in IVIG

Before the introduction of immunoassays for determination of the content of IVIG preparations in antibodies specific to certain bacterial isolates, opsonic assays were mostly used. Opsonic assays provide information on both the amount and the functionality of IgG molecules, i.e. their capacity to bind to an antigen and then induce its phagocytosis via interaction with Fc receptors on phagocytes. Documentation of the opsonic activity of an IVIG preparation against slime-producing *S. epidermidis* is presented in Fig. 2. The introduction of immunoassays (mainly of ELISA type) in analysis of IVIGs for specific antibodies took place in mid 1980s and two approaches have been used for the determination of pathogen-specific antibodies.

In one approach, capsular antigens (polysaccharides or proteins) are extracted without further chemical characterization of each component and antibodies against them are determined by ELISA. Hamill et al. [10] developed such an ELISA method to determine antibody levels to five commonly isolated serotypes of *Streptococcus pneumoniae* in four IVIG preparations. Adjustment of IVIG doses on the basis of specific IgG ELISA titers efficiently protected mice from *S. pneumoniae* infections [10]. Givner [11] studied antibody activity against type III group B *Streptococcus* by comparing the estimated ELISA titers to the opsonophagocytic effect of these antibodies and the protective efficacy in animal models. Differences in activity were determined not only among various IVIG preparations but also among lots from the same manufacturer. Moreover, it was reported that the effect of IVIG using one of the assay methods could not reliably predict activity obtained using the other assays.

With respect to the second approach, in 1994, two research groups reported the determination of antibodies in IVIG preparations against whole bacterial cells by ELISA [12,13]. Given the structure of the outer surface of bacteria, estimated antibodies may recognize lipopolysaccharides, lipid A, capsular antigens, flagella, pili or other structures. Nakae et al. [12] showed the presence of antibodies to clinical isolates of *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* in IVIG and reported that the protective activity against bacterial infection is in proportion to the antibody titer. Hiemstra et al. [13] showed that the levels of antibodies against isolates of *S. aureus*, *E. coli* and *Streptococcus pyogenes* in IVIG preparations were equal to or higher than that of pooled normal human serum, with the exception of an IVIG preparation chemically modified by β-propiolactone treatment. Moreover, they showed a good correlation of ELISA titers with the opsonophagocytic activity against the specific bacterial pathogens, suggesting that antibodies determined by ELISA are functional and this assay can be used to predict IVIG opsonic activity [13]. This is of great importance because opsonic assays are not generally reproducible, due to the fact that phagocytes come from different donors each day and determination of optimal conditions of phagocytosis (e.g. presence or absence of complement) must be performed for each microorganism. Recently, Romero-Steiner et al. [14] reported a standardized opsonophagocytic assay of high repeatability for the measurement of functional antibody activity against *S. pneumoniae* using differentiated HL-60 cells. They also showed high correlation coefficients between opsonophagocytic activity of IVIG and concentrations of specific IgG.

Antibodies to various bacterial isolates (whole bacterial cells) can be determined by a robust enzyme immunoassay of low intra- and inter-variability, which was recently suggested by our research group [15]. Bacterial cells are coated on ELISA microplates and incubated with various dilutions of the sample. The content of IVIG in antibodies to a certain bacterial isolate is defined as the lowest concentration of IVIG giving an absorbance of 0.2 above the background at 490 nm. This method can be applied to the determination of antibodies to a great range of Gram-negative and positive bacterial isolates. Notably, titers of antibodies to *S. aureus* strains were several

orders of magnitude higher than titers of antibodies against the rest of bacterial isolates, as earlier described [12,13]. This is attributed to non-specific interactions of protein A on the cell wall of *S. aureus* with antibodies in IVIG preparations. Significant differences in concentrations of specific IgG were determined not only between different IVIG preparations but also among various lots from the same IVIG preparation. This lot-to-lot variability has been also described by research groups using opsonic assays [16] and may account for the contradictory results of the clinical in vivo studies [7,8].

The high degree of correlation between the content of infused IVIG preparations in pathogen-specific antibodies and their presence in blood sera of treated patients has been recently shown [17]. Selected lots from two IVIG preparations were administered to patients with primary immunodeficiencies and pathogen-specific antibodies were determined in blood sera of patients before and 30 days after IVIG infusion. Sandoglobulin® contained higher amounts of antibodies against *E. coli* and *S. epidermidis* than Gamimmune®, and their infusions resulted in maintenance of levels in the recipients significantly higher than those in healthy individuals and Gamimmune®-treated patients. Correspondingly, estimated titers represented very high amounts of specific antibodies. Both IVIG preparations contained comparable amounts of specific antibodies towards *K. pneumoniae*, that were sufficiently high, since they kept recipients' levels twice above the corresponding levels in healthy individuals of the same median age and sex. Gamimmune® contained higher amounts of *Enterococci* spp.-specific antibodies than Sandoglobulin® and both had comparable levels of antibodies to *S. aureus*. Titers of antibodies to *S. aureus* and *Enterococci spp*. in both preparations seem to represent sufficient but not high enough amount of antibodies, since the respective titers in patients' blood sera were close to those in healthy individuals. The sufficient pathogen-specific antibodies in patients were correlated with their serum IgG2 (the concentration of which is similar to that of healthy individuals) and the good clinical profile of the patients, suggesting satisfactory antibody response to polysaccharide bacterial antigens [17].

3. Antibodies to specific antigenic components of bacteria in IVIG

The presence in IVIG preparations of antibodies specific for secreted bacterial factors, such as exotoxins and superantigens, or components of the outer bacterial surface is of great importance. Such antibodies can neutralize the virulence factors of bacteria or bind to bacteria, hinder their spread, activate the complement and promote their phagocytosis. Reports on the use of immunoassays to determine antibodies to certain antigenic components in IVIG preparations are presented here.

Special attention has been drawn to the lipopolysaccharide (LPS), also known as endotoxin, on the cell wall of Gram-negative bacteria, due to the toxicity of the lipid moiety of the molecule (lipid A) and the immunogenicity (antigenicity) of the polysaccharide side chains (O antigens). Stoll et al. [18] developed an ELISA method for the determination of serum antibodies against common determinants of LPS and, thus, it was possible to define a group of patients with multiple myeloma that were at high risk of contracting Gram-negative infections. Such antibodies were measured in IVIG preparations and therefore the use of IVIG preparations in clinical practice was suggested [18]. In another study, it was shown that measurement of antibodies to LPS antigens was not so sensitive for diagnosis of *Pasteurella multocida* as the determination of antibodies to boiled-cell extract antigen due to the fact that the LPS antigens might have been type-specific [19]. A human intravenous IgG preparation rich in antibodies to different LPS exhibited the same ability to confer passive immunity as a normal polyvalent IVIG preparation [20]. This finding may be explained in light of recent studies suggesting that effective antibodies to LPS mainly belong to immunoglobulin class M [21,22]. ELISA and immunoblot experiments showed that nine different IVIG preparations contained IgG antibodies directed against LPS as well as outer membrane proteins of *Campylobacter jejuni*, one of the most common enterocolitis-causing microorganisms [21]. However, only one immunoglobulin preparation, which was enriched in IgM antibodies, medi-

ated a significant enhancement of *C. jejuni*-triggered production of reactive oxygen metabolites of, as well as killing of *C. jejuni* by human polymorphonuclear leukocytes [21]. Moreover, in a more recent study, mean ELISA levels of antibodies against LPS preparations from *E. coli*, *Klebsiella* and *P. aeruginosa* O serotypes were significantly higher in the IgM fraction of an IgM-enriched product than in 'pure' IVIG preparations [22].

Antibodies against *Clostridium difficile* toxins A and B were detected by ELISA in nine IVIG preparations and all preparations neutralized the activity of *C. difficile* toxins in vitro at IgG concentrations of 0.4–1.6 mg/ml [23]. Administration of IVIG to patients with pseudomembranous colitis not responding to antibiotic therapy elevated the respective specific IgG in serum and helped patients resolve their clinical symptoms (diarrhea, abdominal tenderness, and distension) [23].

Superantigens are microbial proteins capable of activating a large number of T cells, thereby causing an excessive release of inflammatory cytokines. ELISA and Western blot assays revealed high concentrations of antibodies in IVIG against eight different staphylococcal superantigens [24]. The presence of such superantigen-specific antibodies accounts for the fact that the IVIG preparation inhibited in vitro stimulation of human peripheral blood T cells by the staphylococcal toxins [24]. In accordance to these findings, Norrby-Teglund et al. [25,26] showed by immunoblot analysis that IVIGs contain antibodies against a broad variety of superantigens of group A *Streptococcus* that neutralize the superantigenic stimulatory activity and are transferable to the plasma of recipients. However, not all superantigen-specific antibodies had neutralizing activity, i.e. although high titers of antibodies to superantigen A were determined, the inhibitory activity was low [26]. Furthermore, significant variations of neutralizing activity against purified superantigens, streptococcal pyrogenic exotoxins (Spe) and superantigens present in culture supernatant of clinical group A streptococcal isolates were observed among different IVIG preparations and different lots from the same IVIG brand [27]. Recently, the relative avidities for Spe A and B of human IgG antibodies were determined in IVIG preparations and in sera from healthy individuals and patients with fatal streptococcal toxic shock-like syndrome and the values were estimated to be between 10^{-7} and 10^{-11} M [28].

Another major virulence factor of group A streptococci is the surface M protein. ELISA experiments showed the presence of high levels of antibodies to type M1 protein in three IVIG preparations and there was no significant observable difference in the levels among the preparations tested and lots from the same preparation [29]. These antibodies enhanced phagocytosis of type M1 strains of *S. pyogenes* and there was a good correlation between ELISA titers and opsonophagocytic activity [29]. This activity, like the superantigen-neutralizing activity, is conferred to patients receiving IVIG therapy.

Slime-producing *S. epidermidis* is responsible for severe infections in immunocompromised patients, particularly in preterm neonates, which are commonly associated with the formation of a biofilm on the surface of prosthetic medical implants. Bacteria in biofilms are protected from phagocytosis and the action of antibiotics. A sulfated polysaccharide with molecular mass of 20-kDa (20-kDa PS) has been recognized as the major polysaccharide component and antigenic determinant of *S. epidermidis* extracellular slime layer [30–32]. This polysaccharide consists of glucosamine and glucose linked by α-glycosidic bonds. A highly precise and repeatable enzyme immunoassay was developed to determine quantitatively the levels of antibodies against the 20-kDa PS of *S. epidermidis* slime [33]. Results were expressed in relation to rabbit polyclonal antibodies against 20-kDa PS of high reactivity and specificity, and it was agreed that they contained 1000 units of 20-kDa PS specific IgG per mL. As shown in Fig. 3, the amount of 20-kDa PS specific antibodies found in 27 lots of an IVIG preparation (Sandoglobulin®) correlated well with their in vitro opsonic activity against slime-producing *S. epidermidis*.

The majority of lots (75%) having titers higher than 200 units/ml showed significant opsonic activity (50–75%) towards slime-producing *S. epidermidis* [33]. Such IVIG lots were administered

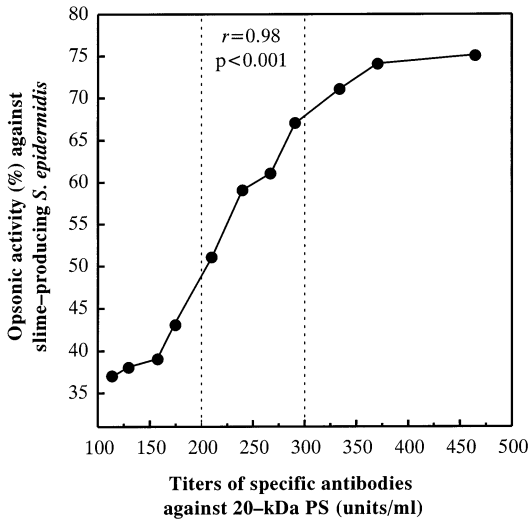

Fig. 3. Diagram showing that antibody titers towards the 20-kDa PS of slime-producing *S. epidermidis* determined by ELISA in various Sandoglobulin® lots are correlated with the opsonic activity of these IVIG lots against slime-producing *S. epidermidis*.

as a prophylactic agent to low-birth weight (lower than 1700 g) preterm neonates immediately after birth. The levels of total and 20-kDa PS specific IgG in neonates' blood sera were significantly higher than those found in the control group, even 10 days after the last infusion. The rate of slime-producing *S. epidermidis* bacteremia in neonates who received IVIG was also considerably lower than those in the control group.

4. Concluding remarks

Immunoassays have successfully found application in the determination of antibodies specific to bacteria and discrete antigenic components in IVIG preparations and blood sera. The estimated amounts of antibodies represent opsonically active, protective antibodies since most studies show a correlation of ELISA titers with IVIG opsonophagocytic activity or neutralizing activity. Most studies, however, have shown a great variability of titers of specific antibodies among different IVIG preparations and among various lots from the same preparation. This important finding may be ascribed to the different manufacturing procedures used or the variability of donor pools and calls for careful adjustment of the dosage schemes. In vivo studies have confirmed the in vitro results and revealed the protective anti-infective efficacy of IVIG administration provided that appropriate lots are selected or the dosage schemes are adjusted.

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