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An enzyme immunoassay to determine the levels of specific antibodies toward bacterial surface antigens in human immunoglobulin preparations and blood serum☆

F. Lamari [a], E.D. Anastassiou [b], T. Tsegenidis [a], G. Dimitracopoulos [b], N.K. Karamanos [a,*]

[a] *Section of Organic Chemistry-Biochemistry and Natural Products, Department of Chemistry, University of Patras, 261 10 Patras, Greece*

[b] *Department of Microbiology, School of Medicine, University of Patras, 261 10 Patras, Greece*

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Abstract

Human polyvalent intravenous immunoglobulin (IVIG) preparations are used as a complementary aid to the proper antimicrobial treatment of severely septic patients in intensive care units (ICUs) and/or as a prophylactic agent to immunocompromized hosts, particularly prone to bacterial infections. There is skepticism about the usefulness of IVIGs since it is not known whether their administration ensures the enhancement of humoral immune responses by providing a sufficient amount of specific antibodies towards the specified bacterial pathogen to be treated. In this report, a simple and reproducible enzyme-linked immunosorbent assay for determining the content of specific antibodies against bacterial surface antigens in commercially available IVIG preparations is described. The method is also easily applied to determine the amount of bacterial antibodies in blood serum. The levels of specific antibodies toward Gram positive and negative pathogenic isolates often encountered in ICUs were estimated in two IVIG (Sandoglobulin® and Gamimmune®) preparations. Significant differences regarding the content of antibodies to certain clinically bacterial isolates were identified not only between the two IVIG preparations tested, but also among various lots from each IVIG preparation. No significant variation ($P \leq 0.001$) among the bottles derived from the same lot was determined in both preparations. The variation in the levels of specific antibodies in IVIG preparations may be attributed to differences between the donor pools as well as the manufacturing procedure. Application of the method to patients with primary immune deficiencies showed that infusion of highly reactive IVIG preparations enhanced significantly their humoral response toward various pathogens. The results of this study suggest that the content determination of pathogen-specific antibodies in IVIG preparations before administration may be of great importance for treating bacterial infections.

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* Corresponding author. Tel./fax: +30-61-997153.

E-mail address: n.k.karamanos@upatras.gr (N.K. Karamanos)

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1. Introduction

In the last decade successful implementation of anti-infective therapy has become increasingly difficult. Traditionally known microbial organisms find ways to evade the antibiotic effect of drugs, new infectious agents emerge, and hospitalized patients become highly susceptible to many infectious agents due to their immunocompromized status. Thus, the pattern of infectious diseases changes incessantly [1,2]. Among other uses, commercially available human polyvalent intravenous immunoglobulin (IVIG) is administered as a prophylactic agent against infection to patients with primary and acquired immune deficiencies and as a complementary aid to the proper antimicrobial therapy of septic patients [3,4]. The initial and, indeed, the primary use of IVIG is to provide specific antibodies against infectious microorganisms enhancing opsonization and killing of the pathogen [5,6].

IVIG preparations constitute pooled normal intact polyspecific IgG obtained from several thousand healthy donors and are produced by a process of multiple alcohol precipitation, centrifugation, enzymic and/or chemical treatments, depending on the manufacturer. It has been demonstrated that IVIG contains significant levels of antibodies toward various pathogenic bacteria (for an excellent review see Ref. [7]). It has been reported [8], however, that opsonic activity of commercially available IVIG preparations was significantly affected by the manufacturers' donor pool and that pathogen-specific opsonic antibody activity is highly variable for several pathogens.

In vivo studies led to contradictory results about the efficacy of IVIG in prophylaxis/treatment of bacterial infections [3,9]. There are indications [10] that this discrepancy stems from the fact that the composition of the administrated IVIG preparations in terms of specific antibodies present is not known, since it has not been recorded by an accurate analytical method.

The need for screening of IVIG preparations for the content of pathogen-specific antibodies, prompted us to develop a simple and rapid enzyme-linked immunoassay to determine the reactivity of human polyvalent immunoglobulin preparations with surface antigens of pathogenic bacteria most often isolated from clinical specimens.

2. Experimental

2.1. Materials and bacterial isolates

Gram positive and negative bacteria (*Enterobacter cloacae*, *E. aerogenes*, *Klebsiella pneumoniae*, *K. oxytoca*, *Pseudomonas aeruginosa*, *Acinetobacter anitratus*, *Esherichia coli*, *Serratia liquefaciens*, *Staphylococcus epidermidis*, *S. aureus*, *S. saprophyticus*, *S. haemolyticus* and *S. scleiferi*) most commonly encountered in patients hospitalized in the ICU at the University Hospital of Patras, were isolated and identified from blood cultures according to protocols routinely used in the Microbiology Laboratory. Eight different lots of Sandoglobulin® (7S intact IgG isolated by precipitation at pH 4 and pepsin treatment, Novartis, Switzerland) at different concentrations (0.03 and 0.06 g/ml) and seven lots of Gamimmune® (7S intact IgG treated by diafiltration at pH 4.25, Bayer, USA) at a concentration of 0.05 g/ml were kindly provided from Novartis (Hellas) S.A.C.I. and Professor F. Kanakoudi-Tsakalidou (A′ Pediatric Unit, 'Ippokratio' General University Hospital, Thessaloniki), respectively. Bovine serum albumin (BSA), peroxidase H-conjugated rabbit anti-human IgG and *ortho*-phenylenediamine tablets of 1 mg were obtained from Sigma (St. Lious, MO, USA). All other chemicals used were of analytical grade.

In order to examine whether the enzyme immunoassay can be used for determination of pathogen-specific antibodies in blood serum, an in vivo study was carried out by infusion of Sandoglobulin® to a patient with X-linked agammaglobulinemia. Blood sera were collected before and one month after the infusion and taken for analysis of pathogen-specific antibodies.

2.2. Enzyme-linked immunosorbent assay

ELISA was performed on sterile 96-well round-bottomed microplates. Suspensions of various bacterial species that had an original optical density equal to 1.0 (path length 1 cm) at 600 nm were diluted 1:40, 1:30, 1:10 and 1:3 with phosphate-buffer saline (PBS). The plates were coated with the bacterial suspensions (100 μl/well) at 4°C for 16 h and were washed with PBS–1‰ (v/v) Tween 20–0.4 M NaCl (110 μl/well). Unspecific binding was blocked by incubating them with a 3% (w/v) solution of highly purified BSA in PBS (200 μl/well) at 37°C for 1 h. The microplates were washed three times with PBS–Tween and incubated with various dilutions of IVIG (1:100, 1:30, 1:10 and 1:3) or blood sera (1:500, 1:100, 1:50) at 37°C for 1 h. Following three washings, peroxidase H-conjugated rabbit anti-human IgG, diluted 1:4000 with PBS, was used as the second antibody and incubated at 37°C for 1 h. The color was developed by adding 0.4 mg/ml *ortho*-phenylenediamine in 0.1 M citric acid in the presence of 12% (v/v) H_2O_2 as enzyme substrate. The mixture was incubated for 15 min at room temperature in the absence of light. The reaction was terminated by adding 1 M H_2SO_4. The absorbance was measured at 490 nm in a Molecular Devices E-max photometer. Calibration and validation of results were performed using the SOFT max PRO software (version 1.2.0).

As a control for non-specific binding, the microplates were coated only with BSA and the procedure continued as described above. The blank values obtained in each ELISA microplate were always automatically subtracted from those obtained in the samples, using the same version of SOFT max PRO software. The steps of the ELISA procedure followed to determine specific IgG in IVIG preparations and blood sera are summarized in Appendix A.

3. Results

3.1. Determination of optimum ELISA conditions and quantification

In order to determine the optimum coating dilution of bacteria to the ELISA microplates, various dilutions (1:40, 1:30, 1:10 and 1:3) of bacteria derived from an original suspension ($A_{600} = 1.0$) were used to coat the microplates and, following the described protocol, they were incubated with various dilutions of Sandoglobulin® and absorbance was measured at 490 nm. The absorbance values obtained for each IVIG dilution was plotted against the various bacterial dilutions. As shown in Fig. 1, the region between 1:10 and 1:40 falls in the linear range of the reactivity curves, and it was therefore decided that all subsequent experiments would be performed at the optimum dilution of 1:30 for coating bacteria to ELISA microplates. Owing to the lack of standard preparations for most of the surface antigens, it is not possible to make an exact quantitative measurement of specific antibodies,

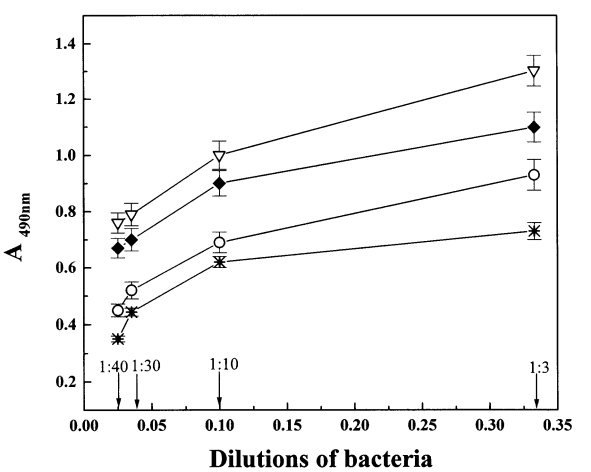

Fig. 1. Determination of the optimum bacterial coating dilution on ELISA microplate. Coating was performed with various dilutions (1:40, 1:20, 1:10, 1:3) of bacteria derived from an original suspension with $A_{600} = 1.0$. Each bacterial dilution was incubated with various Sandoglobulin® dilutions (▽, 1:3; ◆, 1:10; ○, 1:30; ∗, 1:100) and the reaction was recorded by measuring the absorbance at 490 nm. Each point represents the average ± S.D. of six determinations in triplicate per strain. The diagram shows the behavior of *E. cloacae*. All other bacteria tested showed similar patterns. 10 strains of all bacteria were studied.

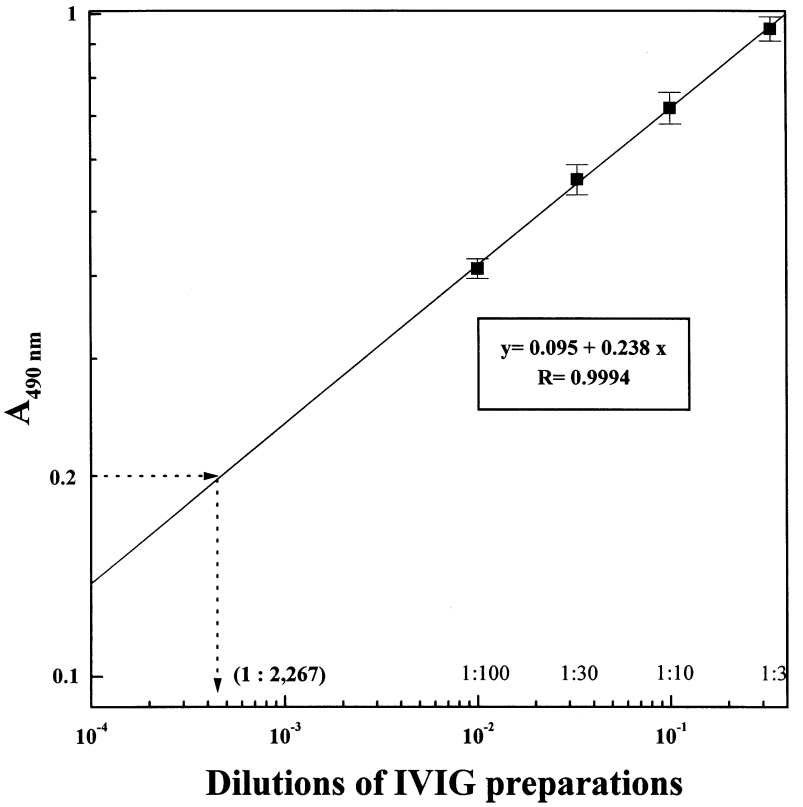

Fig. 2. Representative log–log calibration curve used for the determination of the specific antibody concentration in the IVIG preparations. Cells, *E. cloacae* in this case, were diluted 1:30 in PBS, coated to the ELISA microplate and incubated with various IVIG dilutions (1:100, 1:30, 1:10, 1:3). A_{490} was then plotted vs. the various IVIG dilutions in a log–log diagram. Extrapolation of the curve to the absorbance value of 0.2 gives a dilution of 1:2267 for the IVIG preparation. By multiplying this dilution with the IVIG concentration (0.03 g/ml), i.e. 0.03 g/ml × 2267, the titer in U/ml of specific IgG to surface antigens of *E. cloacae* isolate (68 U/ml) is estimated.

but only semi-quantitative determinations. Therefore, the content of IVIG or otherwise referred to as titer of specific antibodies to certain bacterial surface antigens was defined as the lowest concentration of IVIG giving an absorbance of 0.2 at 490 nm, as suggested by Kemeny [11]. Particularly, the absorbance obtained at 490 nm was plotted against the various IVIG dilutions in a log–log diagram. The certain factor of dilution of IVIG that results in an A_{490} value of 0.2, above the background, was multiplied by the concentration of the IVIG preparation in g/ml. The resulting value in U/ml is the titer of the pathogen-specific antibodies in the IVIG preparations (see Fig. 2 for a more explicit demonstration).

3.2. Method's quality parameters

Under the conditions described, the intra-variation from well to well in the same ELISA microplate, as calculated by analysis for 10 wells in triplicate (treated in exactly the same way), was very low giving a coefficient of variation (CV) of 3.0%. Inter-variation of the method was examined by performing the same immunoassay on different days and/or by different scientists. Virtually low variations were produced (a CV of 2.7% for different scientists and 3.6% for different days and/or the same scientists). The low intra- and inter-assay variation reveal the high degree of repeatability, reproducibility and robustness of the immunoassay.

3.3. Screening of IVIG preparations for pathogen-specific antibodies

In order to examine whether the levels of specific antibodies in the IVIG preparations depend on the type of microorganism, the enzyme-linked immunosorbent assay was applied to a great range of clinical bacterial isolates. For this reason a Sandoglobulin® lot was screened for the content of specific antibodies against the commonest highly resistant Gram negative bacteria and various staphylococci species isolated from blood cultures of patients hospitalized in the ICU. Two to ten strains of each bacterial species were tested. All bacteria sufficiently coated the microplates under the particular coating conditions without any manipulation. The titers of specific antibodies to the various pathogens are summarized in Table 1. It can be seen that the content of antibodies in a certain IVIG lot toward bacterial surface antigens is greatly dependent on the specific bacterial species. However, no significant variation ($P \leq 0.001$) among 20 bottles derived from the same lot was determined for all bacteria tested.

In order to examine the lot-to-lot variation in the IVIG preparations, eight Sandoglobulin® lots and seven Gamimmune® lots were screened for the levels of antibodies toward surface antigens of the clinically isolated *E. coli*, *P. aeruginosa*, *K. pneumoniae* and various *Enterococci* species. The lot-to-lot variation of the titers of pathogen-specific antibodies in both IVIG preparations was remarkable (Fig. 3). Although all Sandoglobulin® lots contained high titers of specific antibodies to *E. coli*, such antibodies were not detected in the Gamimmune® lots examined. Sandoglobulin® contained higher levels of antibodies towards *P. aeruginosa*. The content of specific antibodies to *K. pneumoniae* in both preparations were comparable, whereas those to various *Enterococci* species were significantly variable both in Gamimmune® and Sandoglobulin®.

3.4. Application to the determination of specific antibodies in blood sera

One of the most important assets of the method developed is that it can easily be applied for the determination of antibodies in blood sera so as to monitor the progress of an infection or the effectiveness of the administrated IVIG preparation as a prophylactic agent or adjunctive therapy. Sandoglobulin® was administered (0.6 g/kg/month) as substitution therapy to a patient with primary immune deficiency at an infusion rate of 2.5 ml/min and antibodies against *P. aeruginosa* and *K. pneumoniae* were determined in blood sera before and 1 month after the infusion.

The levels of specific antibodies were expressed as the lowest concentration of IgG in blood sera giving an absorbance of 0.2, above the background, at 490 nm. Total IgG in blood sera were determined by a standard nephelometric method. One month after Sandoglobulin® infusion, IgG specific to a clinical isolate of *K. pneumoniae* increased from to 3.4 to 5.94 U/ml (75% increase), while IgG specific to *P. aeruginosa* from 4.23 to 8.340 U/ml (97% increase). The levels of total IgG also followed an increasing pattern; an increase of 38% (from 4.6 to 6.2 g/l) was noted during the same period.

Table 1
Content of antibodies in a Sandoglobulin® lot toward surface antigens of common bacterial isolates encountered in an ICU

Gram negative bacteria	Titer (U/ml)	Gram positive bacteria	Titer (U/ml)
S. liquefaciens	132	*S. epidermidis*	217
A. anitratus	105	*S. aureus*	135
P. aeruginosa	93	*S. saprophyticus*	84
K. pneumoniae	75	*S. haemolyticus*	75
E. aerogenes	72	*S. scleiferi*	66
E. cloacae	68		
E. coli	57		
K. oxytoca	42		

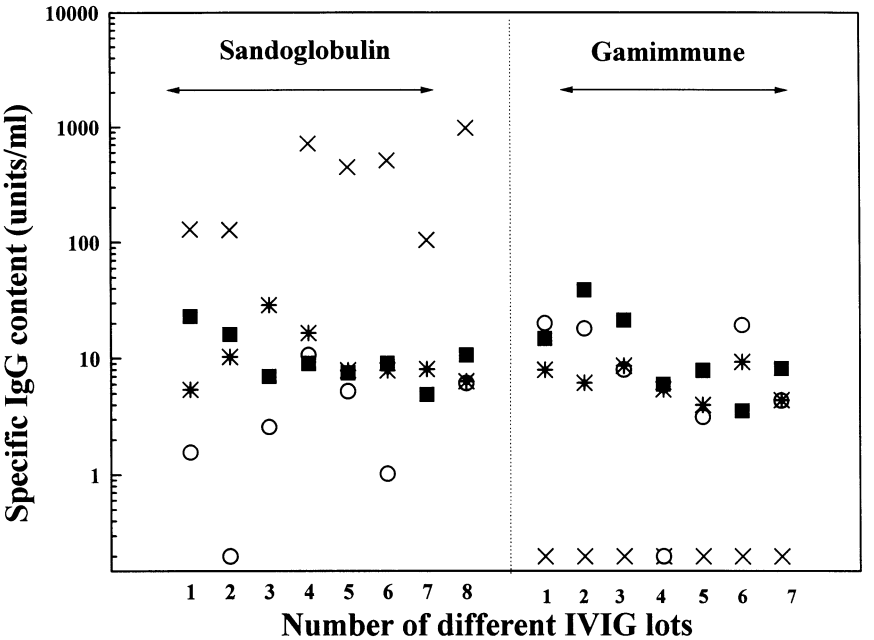

Fig. 3. Screening of two IVIG commercially available preparations (Sandoglobulin® and Gamimmune®) for their content in specific IgG to *E. coli* (×), *P. aeruginosa* (∗), *K. pneumoniae* (■), *Enterococci* spp (○). Determinations were performed using the described protocol (see Appendix A). The data show that there is a significant lot-to-lot variation in the levels of specific antibodies in both IVIG preparations tested, as well as a pathogen-dependent level of their specific IgG.

4. Discussion

Intravenous immunoglobulins exert their anti-infective function through a variety of mechanisms. The most obvious mechanism is the passive administration of antibodies specific to bacterial surface antigens which facilitate the inhibition of microbial attachment, complement activation and opsonization. There is also evidence regarding the existence of neutralizing antibodies to infectious antigens, toxins and bacterial superantigens in different IVIG preparations. 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 lessen its consequences [6,12].

In previous studies where specific antibodies to bacteria have been determined, the levels of specific antibodies have been expressed in relation to those present in pooled normal human serum [13,14]. Semi-quantitative screening of IVIG preparations for antibodies specific to bacterial surface antigens is carried out by the suggested method without requiring normal human serum. The proposed set-up is simple, robust and reproducible.

The content of the two IVIG preparations examined for specific antibodies against several bacteria tested is shown to be pathogen-dependent. Thus, specific antibodies to surface antigens of *E. coli*, for example, were not detected in Gamimmune®, whereas they were present in large amounts in Sandoglobulin®. This finding may probably be attributed to differences in the donor pools, since Gamimmune® is derived from a USA donor pool and Sandoglobulin® from a Central European donor pool.

IVIG contains a wide variety of antibodies but the preparation stability and the variation of IgG titers depend on the number of donors. Results show considerable lot-to-lot variation in specific antibodies' levels (up to 11-times difference in the

titer for a certain pathogen) in both IVIG preparations. Larger donor pools may be necessary in order to circumvent this problem. Similarly, a high degree of variation has also been observed in a study investigating levels of antibodies to enteroviruses in IVIG where differences in titers did not correlate to enterovirus epidemiology at the time that the blood was collected [15]. A number of studies provide evidence that the ELISA titers are indicative of their opsonic activity, reflecting their ability to promote phagocytosis, clearance of bacteria, and protective activity against bacterial infections in mice [13,16,17]. It is, therefore, concluded that careful screening of each IVIG lot against the infectious agents will give important information to the clinician since one can be aware of the activity of the lot before administration. Thus, the ideal practice would be to test IVIG preparations using the patient's bacterial isolate when IVIG is going to be administrated as a therapeutic agent. This knowledge will definitely help clinicians to adjust the administration scheme according to the needs in each particular case ensuring the effectiveness of the treatment.

The suggested immunoassay also allows the determination of antibody titer in blood sera. The analysis can be helpful in monitoring the course of an infection or in evaluating the efficacy of IVIG administration as a prophylactic or therapeutic agent. An interesting application in a patient with primary immune deficiency has been presented. Infusion of Sandoglobulin® resulted in the enhancement of humoral response, since the specific IgG titers to *P. aeruginosa* and *K. pneumoniae* a month after the infusion were still considerably higher than those before the infusion. This is of great importance since such patients are antibody-deficient.

In order to draw conclusions about the efficacy of the IVIG preparations a large number of IVIG preparations should be analyzed for a wide range of pathogens and the titer to be related with their in vivo reactivities. Although the present results concern determination of specific antibodies, further studies to investigate other mechanisms through which IVIG exert their action, such as those including interactions with the cytokine network [3], are in progress in our laboratories.

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Appendix A. Steps followed to estimate pathogen-specific IgG titers in IVIG preparations by ELISA.

1. Bacterial isolates from blood cultures, grown in an appropriate culture medium are centrifuged and then suspended in PBS so that A_{600} nm is 1.0.
2. Bacteria at the dilution of 1:30 in PBS are coated to the 96-well microplates for 16 h overnight at 4°C. Blank wells are coated only with PBS.
3. Microplates are washed three times with PBS–Tween at room temperature.
4. The solution of BSA in PBS is added to all wells and the microplates are incubated for 1 h at 37°C.
5. The microplates are washed three times with PBS–Tween, as in step 3.
6. Various dilutions of IVIG preparations or blood sera in PBS are added to bacteria-coated and blank wells and the mixtures are incubated for 1 h at 37°C.
7. The microplates are washed again, as in step 3.
8. Goat anti-human IgG-peroxidase H at a dilution of 1:4000 in PBS is added and the mixtures are incubated for 1 h at 37°C.
9. The microplates are washed again, as in step 3.
10. The solution containing the substrate for the enzyme is added. This solution is prepared just prior its use by dissolving 1 mg of *ortho*-phenylenediamine in 2.5 ml 0.1 M citric acid, containing 12% H_2O_2 (v/v).
11. Incubation with enzyme substrate is carried out in the absence of light at room temperature for exactly 15 min.

12. The reaction is stopped by the addition of 1 M H_2SO_4. The absorbance at 490 nm is measured and the blank values are subtracted from the corresponding reaction wells.

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