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Determination of slime-producing *S. epidermidis* specific antibodies in human immunoglobulin preparations and blood sera by an enzyme immunoassay Correlation of antibody titers with opsonic activity and application to preterm neonates

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Abstract

Slime-producing *Staphylococcus epidermidis* is responsible for severe infections in immunocompromised patients and, particularly, in premature infants who are transiently deficient in IgG. 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. The presence of adequate amounts of antibodies to 20-kDa PS in patients' sera would be of importance to prevent or treat slime-producing *S. epidermidis* bacteremia. Administration of intravenous immunoglobulin (IVIG) is considered to be a reasonable IgG replacement therapy and has been widely used to prevent or treat neonatal sepsis. Clinical trials have shown conflicting results on the efficacy of IVIGs and this phenomenon has been attributed to the variability of IVIG preparations in the content and opsonic activity of IgG against microorganisms of clinical importance. Monitoring of antibodies to distinct bacterial macromolecules, which are species-specific and responsible for bacterial infections, has not been performed previously. 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. 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*. Sandoglobulin® lots with titers higher than 200 units/ml of 20-kDa PS specific IgG were administered as a prophylactic agent to low-birth weight (lower than 1700 g) preterm neonates

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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. The results of this study suggest that specific IgG titers estimated by the developed enzyme immunoassay may well be indicative of the IVIG opsonic activity against slime-producing *S. epidermidis*. Furthermore, administration of Sandoglobulin® with titers higher than a cut-off value of 200 units/ml may significantly protect preterm neonates against slime-producing *S. epidermidis* bacteremia.

Keywords: Enzyme immunoassay; Intravenous immunoglobulin; *Staphylococcus epidermidis*; Slime; Polysaccharide antigens; Preterm neonates

1. Introduction

Although advances in neonatal intensive care have led to increased survival of preterm neonates, infection remains an important cause of morbidity and mortality among infants [1]. Gram-positive, coagulase-negative staphylococci, primarily *Staphylococcus epidermidis*, have been identified as a major cause of late-onset neonatal sepsis [2–5]. Utilization of intravenous catheters [6], parenteral nutrition, and other indwelling prosthetic devices, predispose the hosts to coagulase-negative staphylococcal infections [4,5,7]. *S. epidermidis* has the capacity to colonize medical devices and form biofilms [8,9]. The production of a highly adhesive extracellular material, known as slime, promotes interbacterial shielding and adherence between bacteria and synthetic devices or biologic tissues. Furthermore, it confers resistance to antibiotics and to phagocytosis [10].

The extracellular layer of slime-producing *S. epidermidis* strains consists of discrete macromolecules [11]. Among them, a sulfated 20-kDa polysaccharide (20-kDa PS) has been identified as the major polysaccharide component of slime (60–65% of slime macromolecules) [12]. Studies with polyclonal rabbit antisera raised against 20-kDa PS and slime have demonstrated that the 20-kDa PS is the principal antigenic determinant of slime [13]. This polysaccharide was found to be immunogenic in humans, since blood sera derived from patients with established slime-producing *S. epidermidis* bacteremia contained approximately 16 times higher titers of 20-kDa PS specific antibodies as compared to those of healthy individuals [13].

Premature neonates are deficient in IgG, since maternal antibodies pass from mother to child primarily in the last trimester of pregnancy. Therefore, administration of intravenous immunoglobulin (IVIG) seems of importance to prevent or treat neonatal sepsis. Clinical studies, however, have led to controversial results concerning the in vivo efficacy of IVIGs [14,15]. It has been shown [16], that protection of animals depends on the amount of *S. epidermidis* specific antibodies present in IVIG preparations, because specific antibodies can enhance opsonization and, thus, promote *S. epidermidis* clearance. A recent study in our laboratories revealed that among different commercially available IVIG preparations, as well as different lots of the same IVIG preparation, exist significant differences regarding the content of antibodies to surface antigens of certain bacterial isolates [17,18]. However, none of the studies conducted so far have examined the variation of specific antibodies against the slime of *S. epidermidis* and its discrete macromolecules.

In light of these data, we report on the development of a precise enzyme immunoassay for screening different IVIG lots for the presence of specific antibodies towards the major antigenic determinant of *S. epidermidis*' slime. The titers of specific antibodies are related to their opsonic activity and their in vivo efficacy to protect preterm neonates against slime-producing *S. epidermidis* infections.

2. Experimental

2.1. Bacterial strains

Slime-producing *S. epidermidis* reference strains 35983 and 35984 obtained from the American

Type Culture Collection (ATCC, Rockville, MD, USA) and two slime-producing clinical isolates obtained from patients with catheter-related bacteremia were used for the isolation of crude slime. Identification of clinical isolates was determined by Gram staining, catalase reaction, tube coagulation tests and, ultimately, by the API Staph-Trac system (Analytab Products, USA). The tube adherence test described by Christensen et al. [19] was used to determine the ability of *S. epidermidis* strains to adhere to polystyrene surface and, subsequently, to produce slime. Stock cultures of all strains were maintained in Tryptic Soy Broth (TSB; BBL Microbiology, Cockeysville, MD, USA) supplemented with 20% glycerol at −70°C without serial passage. For the phagocytosis assay, live microorganisms were suspended in Hank's Balanced Salt Solution (HBSS) containing 0.1% (w/v) gelatin (GHBSS).

2.2. Isolation of crude slime and the 20-kDa PS

Extracellular slime material was obtained as previously described [11]. Mid-log phase bacterial suspensions were inoculated in TSB and grown for 24 h at 37°C in humidified chambers. Slime was separated from the cells by gentle mechanical shaking with glass beads. The extract was precipitated with a mixture of ethanol, sodium acetate and acetic acid at final concentrations of 80% (v/v), 0.26 and 0.05 M, respectively. The pellets obtained were dissolved in 2 × distilled water and the insoluble material was removed by centrifugation at 27 000 × *g* for 30 min. All the above mentioned steps were performed at 4°C in the presence of proteinase inhibitors.

The 20-kDa PS was isolated as previously described [12]. In brief, aqueous solutions of slime were fractionated by ion-exchange chromatography on DEAE-Sephacel, using a linear NaCl gradient (10 vol.) ranging from 0.2 to 1.0 M. Fractions positive with the anthrone reaction were pooled and concentrated on YM-2 membrane (Amicon), dialyzed against 100 vol. of 2 × distilled water and re-chromatographed on DEAE-Sephacel. Anthrone-positive fractions were then collected, concentrated on YM-2 membrane, and dialyzed against 100 vol. of 0.5 M ammonium formate, pH 7.0. The homogenous polysaccharide population was isolated by gel-permeation chromatography on Sepharose CL-6B ($K_d = 0.6$).

2.3. Preparation of antibodies to 20-kDa PS and slime

Antibodies to 20-kDa PS and slime were raised in 4–5-month-old New Zealand White rabbits weighing 4–5 kg. Animals were kept one per cage under a 07:00 h light on, 19:00 h light off cycle having ad libitum access to food and water. Filter-sterilized antigens were emulsified with equal volumes of Freund's complete adjuvant for the first injection and incomplete Freund's adjuvant for the following challenges, which were performed subcutaneously 2 and 5 weeks later. Each animal was challenged with 0.9 mg of dry weight 20-kDa PS and 6.0 mg of dry weight slime every time. Sera obtained in the seventh week were enriched for immunoglobulins by precipitation with $(NH_4)_2SO_4$ and gel chromatography on Sephadex G-25 (PD-10 prepacked column, Pharmacia, Uppsala, Sweden) as has been previously described [13]. They were ultimately used in PBS in the presence of 0.1% (v/v) Tween-20 at a volume equal to the initial antisera volumes.

2.4. Dot immunobinding assay

Interactions of IVIG with slime and the 20-kDa PS were studied by a dot immunobinding assay. One hundred microliters of 5.0, 1.0, 0.7, 0.5, 0.3 and 0.1 μg/ml of slime and 20-kDa PS were coated to Immobilon-P membrane (Millipore, Waters, Milford, MA, USA) using a 96-well manifold at a tap-rated vacuum for 1–2 min. To prevent non-specific binding of immunoglobulins, membranes were incubated for 1 h with 3% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.3. The blocking solution was removed, membranes were washed with 0.5% (w/v) BSA in PBS containing 1% (v/v) Tween 20 and 0.4 M NaCl. Both slime and 20-kDa PS coated membranes were separately incubated with anti-slime, anti-20-kDa PS sera as well as with Sandoglobulin® for 1 h. Antibodies were used in a 1:4000

dilution in PBS. Membranes were washed five times and incubated for 1 h with the detection antibody (peroxidase H-conjugated goat anti-rabbit IgG or rabbit anti-human IgG). Following treatment with the enhanced chemiluminence reagents (ECL, Amersham, Bucks., UK) and pilot film exposure experiments, the membranes were ultimately exposed to Agfa Curix XP film (Agfa Co., Belgium) for 10 s. All steps were performed at room temperature.

2.5. Enzyme immunoassay to determine 20-kDa PS antibody titers in IVIG lots and blood sera

An enzyme-linked immunosorbent assay (ELISA) was developed to determine 20-kDa PS specific IgG in IVIG and blood sera. Sterile 96-well round bottomed microplates were coated with a 5.0 μg/ml solution of 20-kDa PS in a carbonate-bicarbonate buffer, pH 9.6 (100 μl/well), at 4°C for 16 h. The plates were washed three times with PBS containing 1% (v/v) Tween 20, 0.5% (w/v) BSA, and 0.4 M NaCl (PBS-Tween) (120 μl/well). Non-specific binding was blocked by incubation with a 3% (w/v) solution of BSA in PBS (200 μl/well) at 37°C for 1 h. After three washings (220 μl/well), microplates were incubated at 37°C for 1 h with anti-20-kDa PS serum diluted 1:8000, 1:16 000 and 1:32 000 in PBS as well as neonatal sera diluted 1:2000 in PBS, or IVIG lots diluted 1:5000. Sandoglobulin® lots were kindly provided from Novartis Hellas SACI. Plates were washed with PBS-Tween for three times. Peroxidase H-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO), diluted 1:2000, and/or, rabbit anti-human IgG (Sigma), diluted 1:4000 in PBS were used as detection antibodies. After incubation at 37°C for 1 h and thorough washings, color was developed by adding 100 μl/well of a citrate buffer solution, pH 5.0, containing 0.4 mg/ml *ortho*-phenylenediamine dihydrochloride in 0.1 M trisodium citrate and 0.03% (v/v) H_2O_2. The mixture was incubated for 15 min at room temperature in the absence of light. The reaction was terminated with 1 M H_2SO_4 and the optical density 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, some wells in every microplate were coated only with BSA and incubated with the samples (blood sera or IVIG) tested. The respective values were always automatically subtracted from those obtained from the incubation of PS-coated wells with samples.

2.6. Competitive ELISA

The specificity of rabbit antibodies and IVIG against 20-kDa PS was studied by competitive ELISA. In brief, rabbit anti-20-kDa PS serum at a final dilution 1:8000 and IVIG at 1:2000 were incubated with 20-kDa PS in capped polypropylene tubes at different concentrations for 16 h at 4°C. Microplates were coated with 20-kDa PS, washed, and incubated with BSA, as stated above. After three washings to remove unbound BSA, microplates were incubated with the antisera-PS mixtures (100 μl/well). The amount of antibodies that bound to PS was detected with peroxidase H-conjugated goat anti-rabbit IgG and/or rabbit anti-human IgG.

2.7. Phagocytosis assay

The opsonic activity of rabbit anti-20-kDa PS antibodies, rabbit anti-slime antibodies and different lots of Sandoglobulin® was determined by measuring their ability to promote phagocytosis of bacteria by human granulocytes. Polymorphonuclear leukocytes were isolated from fresh buffy coats of healthy individuals (kindly provided by the Blood Bank of 'St. Andrew' General Hospital, Patras) by ficoll-hypaque density centrifugation. Remaining erythrocytes were lysed using a 0.2% (w/v) NaCl solution for 90 s and centrifuged (800 rpm, 10 min). This procedure was repeated three times and the final cell pellet was washed once with ice-cold GHBSS and adjusted to the appropriate concentration (25×10^6 cells/ml) in GHBSS. *S. epidermidis* cells (ATCC 35983) were opsonized with Sandoglobulin® (10 mg/ml), anti-20-kDa PS, and anti-slime antibodies at 4°C for 30 min and then presented to human

granulocytes at 37°C for 90 min. The final cell-bacteria suspension consisted of 2×10^6 granulocytes/ml, 2×10^6 bacteria/ml, and the tested antibodies in concentrations of 0.8 mg/ml for IVIG and 8% (v/v) for rabbit anti-20-kDa PS, anti-slime or normal rabbit sera. Control experiments measured the bactericidal activity of granulocytes in the absence of IVIG or specific antibodies. At the end of the incubation period, samples were diluted in GHBSS and cultured on blood agar plates to quantify viable bacterial colonies. Cultures were performed for 18–24 h at 37°C, colonies were counted, and phagocytosis was determined as the per cent decrease of viable cells. Each experiment was performed five times in triplicate. As an internal control, opsonization of *S. epidermidis* (ATCC 35983) by anti-20 kDa PS serum was included in every experiment. Results were expressed in relation to the opsonic activity of anti-20 kDa PS serum.

2.8. Clinical study

Fifty-two preterm low birth weight (< 1700 g) neonates hospitalized in the Neonatal Intensive Care Unit of 'Aghia Sophia' Children's Hospital entered the present study within the first 24 h of their life. Informed parental consent was obtained for all infants. Commercially available polyvalent immunoglobulin (Sandoglobulin®) was infused to 26 neonates (400 mg/kg of body weight per day) for 4 consequent days (IVIG group) starting on the first day after birth, at a flow rate of 2.8 mg/min, while the remaining 26 infants (control group) did not receive IVIG. Sera obtained before infusion (day zero), on the fourth (day 1-post), eighth (day 5-post) and eighteenth day (day 15-post) were measured for total and 20-kDa PS specific IgG. Total IgG titers were determined by an IgG turbidimetric kit (The Binding Site Ltd., Birmingham, UK). Sandoglobulin® (7S intact IgG isolated by precipitation at pH 4) from the same lot was administered to all neonates. A small quantity from every bottle used for infusion was evaluated for total and specific IgG to 20-kDa PS.

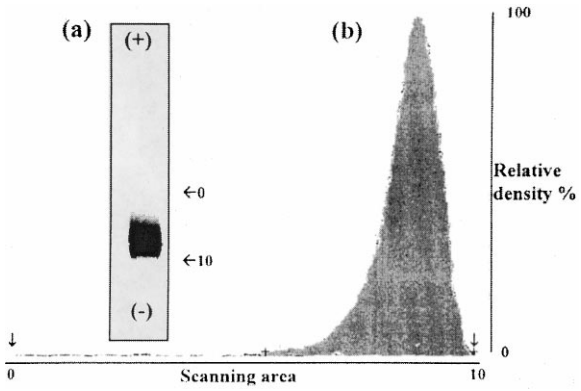

Fig. 1. Determination of IgG homogeneity and purity in Sandoglobulin®. (a) Agarose gel electrophoresis of Sandoglobulin®. (b) Distribution of IgG population determined by fine scanning between the area indicated by the arrows (0–10).

2.9. Analysis of data

Statistical analysis of data was performed by *t*-test and two-way completely randomized analysis (ANOVA), using the Microcal Origin software, version 5.0.

3. Results

3.1. Determination of immunoglobulin classes in Sandoglobulin® preparations

The macromolecular composition of Sandoglobulin® preparation was examined. As shown in Fig. 1(a), Sandoglobulin® migrated as a single band in an agarose gel exhibiting an homogenous IgG population as the major immunoglobulin. Fine scanning of this band showed the absence of other protein/glycoprotein contaminants at a level of limit ca. 2% (Fig. 1(b)). The electrophoretic results were verified with a nephelometric method used routinely in the University Clinical laboratory. Determination of immunoglobulin content in 27 Sandoglobulin® lots, showed that they contain almost exclusively IgG ($98.94 \pm 1.1\%$), whereas IgM and IgA are present in minute amounts ($0.86 \pm 0.2\%$ and $0.20 \pm 0.05\%$, respectively).

3.2. Reactivity of Sandoglobulin® with 20-kDa PS

The immunoreactivity of Sandoglobulin® with the major constituent of *S. epidermidis*' slime was

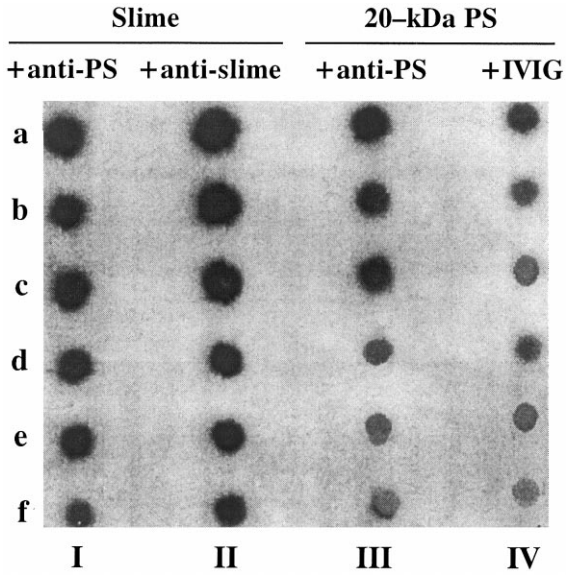

Fig. 2. Dot immunobinding assay to evaluate immunoreactivity of 20-kDa PS with anti-slime serum and interaction of Sandoglobulin® with 20-kDa PS. Slime (I and II) and 20-kDa PS (III and IV) were coated at 5.0, 1.0, 0.7, 0.5, 0.3 and 0.1 μg/ml (a–f, respectively). The membrane was cut into four lanes. Membrane lanes I and III were incubated with anti-20-kDa PS serum, lane II with anti-slime serum and lane IV with Sandoglobulin®. Visualization of spots was performed using chemiluminence and exposure on Agfa Curix XP film for 10 s.

studied using dot immunobinding assay. Study of cross-reactivity of rabbit anti-slime and anti-20-kDa PS sera with their homologous antigens, slime and 20-kDa PS, showed that the 20-kDa PS specific antibodies react with slime to an extent similar to that recorded for slime specific antibodies (Fig. 2, lanes I and II). This is in complete agreement with earlier studies showing that the 20-kDa PS is the major antigenic determinant of slime [13]. A high degree of reactivity between the 20-kDa PS and Sandoglobulin® (lane IV), as compared to that between the 20-kDa PS and its specific antibodies (lane III), was also obtained. From the results it can be deduced that Sandoglobulin® contains a considerable proportion of IgG that recognizes the major 20-kDa PS antigenic determinant of *S. epidermidis'* extracellular slime.

The specificity of Sandoglobulin® binding to 20-kDa PS was evaluated by competitive ELISA. The 20-kDa PS was used as substrate to study the inhibition of the binding with its homologous antisera and Sandoglobulin®. Direct reaction of anti-20-kDa PS serum at a final dilution 1:8000 with 20-kDa PS results in absorbance of 1.2 units at 490 nm. The same absorbance value was obtained using a dilution of 1:2000 of the tested Sandoglobulin® lot. Separate incubations of anti-20-kDa PS antibodies and Sandoglobulin® with increasing concentrations of 20-kDa PS reduced the amount of antibodies in the mixtures that could interact with the PS on the microplate, and, therefore, the recorded absorbance (Fig. 3). This approach revealed that antibodies towards the 20-kDa PS, detected in Sandoglobulin® with dot-binding immunoassay, are specific to this polysaccharide antigen. Binding inhibition by 50% of anti-PS serum and Sandoglobulin® were observed using 3.2 and 5.6 μg/ml of 20-kDa PS, respectively. These findings further suggested that significant amounts of antibodies specific to 20-kDa PS are present in the Sandoglobulin® preparations.

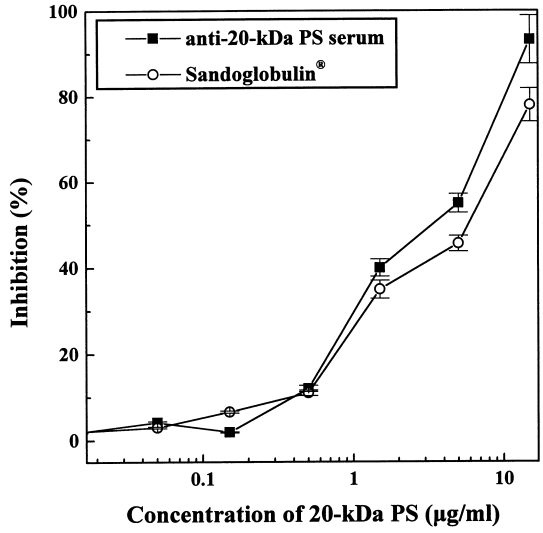

Fig. 3. Displacement curves from competitive ELISA experiments. Inhibition between anti-20-kDa PS serum and its homologous antigen (■) and between Sandoglobulin® and 20-kDa PS (○). Every point represents the average value ± S.D. of four experiments in triplicate.

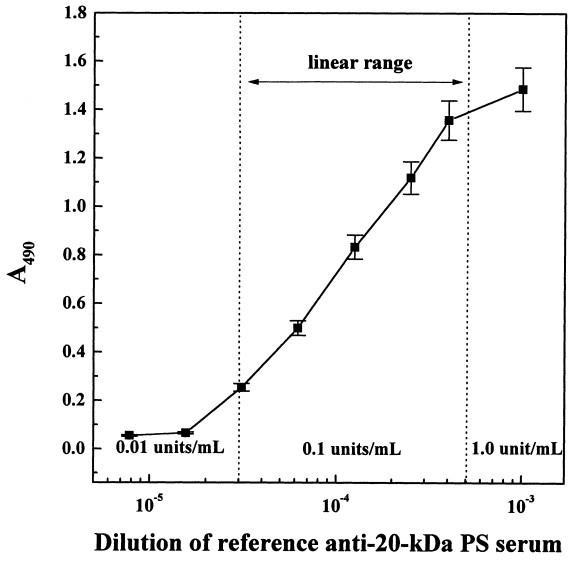

Fig. 4. Calibration of the reference curve used for determination of 20-kDa PS-specific IgG in various Sandoglobulin® lots. The curve was obtained using 20-kDa PS coated at 5.0 μg/ml and anti-20-kDa PS serum at dilutions ranging from 1:1000 to 1:128 000. Each point of the curve represents the average values of six experiments performed in triplicate.

3.3. Determination of 20-kDa PS-IgG titers in Sandoglobulin® lots

Antibodies to 20-kDa PS were measured in a number of different lots from the same IVIG preparation with the developed enzyme immunoassay. The content of antibodies to *S. epidermidis* polysaccharide in various Sandoglobulin® lots was expressed in relation to anti-20-kDa PS sera. A reference curve was constructed by plotting the optical density at 490 nm versus serial dilutions of antisera in a semi-log graph (Fig. 4). The sigmoid pattern of the curve revealed very high reactivity of anti-20-kDa PS serum with its homologous antigen.

For quantification of the amount of specific antibodies towards the 20-kDa PS, it was assumed that the reference serum has 1000 units/ml. This assumption reflects that the value of 1.0 unit/ml corresponds to 1/1000 dilution of reference antisera. Reactivity of various IVIG lots was determined by selecting dilutions that generated absorbance values within the linear part (0.031–0.500 units/ml, $y = (4.474 \pm 0.165) + (0.930 \pm 0.038)\log x$, $R = 0.997$) of the reference curve. The X-intercept of the absorbance values in units/ml was multiplied by the appropriate dilution factor, for instance $\times 4000$ for a 1:4000 dilution of a 0.3 g/l Sandoglobulin® solution, and the titers were expressed in units/ml of the preparation. As an internal control, a reference curve was created in every microplate using the dilutions 1:32 000, 1:16 000 and 1:8000 of the reference anti-20-kDa PS serum, which correspond to the linear part of the curve (Fig. 4).

Under the conditions described, the intra-variation from well to well in the same 96-well microplate, as calculated by analysis of ten wells in triplicate (treated in exactly the same way) was very low, giving a coefficient of variation (CV) of 3.0%. Inter-assay variation was calculated by estimating titers of 20-kDa PS specific IgG in the same sample on different days and by different scientists. Virtually low variations were produced for different scientists (CV 4.3%) and for different days and the same scientist (CV 6.4%). These results suggest that the method is repeatable and reproducible.

Twenty-seven Sandoglobulin® lots of 0.3 g/l concentration were tested with the developed immunoassay. The estimated titers ranged from 113.8 to 465.24 units/ml. The mean titer was 241.94 ± 78.59 units/ml (CV 32.5%), while at the confidence level of 95% ($P = 0.05$) the average value was 241.94 ± 29.65. The distribution of the 20-kDa PS antibody titers in Sandoglobulin® lots is shown in Fig. 5. The majority of Sandoglobulin® lots (75%) contained specific antibodies against the 20-kDa PS at levels higher than 200 units/ml. A 60% portion showed titers between 200 and 300 units/ml, whereas titers lower than 200 units/ml were estimated in 25% of Sandoglobulin® lots. These results indicate that although the levels of specific IgG against the major polysaccharide determinant of *S. epidermidis*' slime in Sandoglobulin® are lot-to-lot variable, the probability for an analyst or a clinician to receive a lot containing relatively high titers (higher than 200 units/ml) is 75%. Screening of 20 bottles of one particular lot for 20-kDa PS specific antibodies did not show any significant differences.

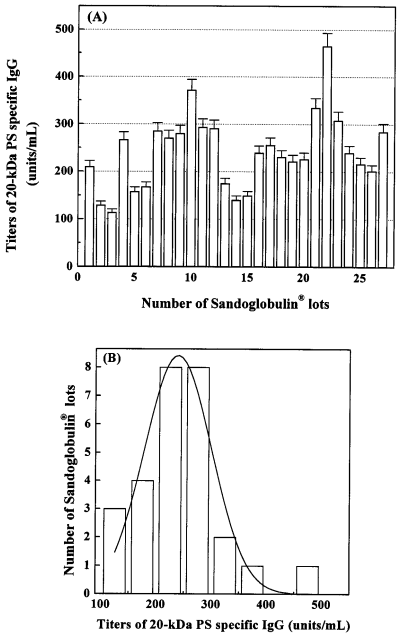

Fig. 5. (A) Distribution of 20-kDa PS-specific antibodies in 27 Sandoglobulin® lots. Results are expressed as arbitrary units (units/ml) in relation to the rabbit anti-20-kDa PS serum. (B) Histogram depicting the frequency of lots with titers within an interval of 50 units/ml.

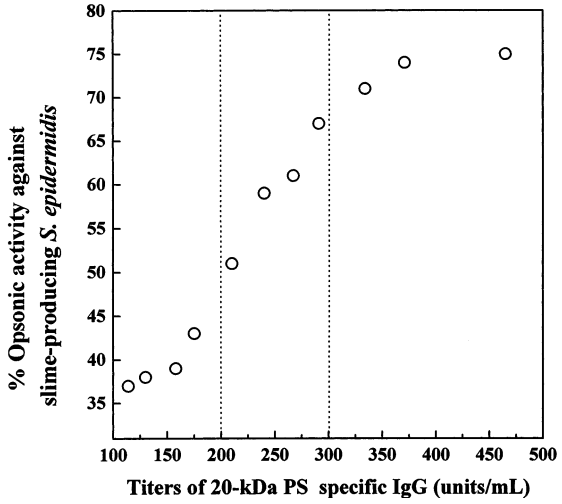

Fig. 6. Correlation analysis of the relation between the content of 20-kDa PS specific antibodies in 11 Sandoglobulin® lots and the activity to promote phagocytosis of slime-producing *S. epidermidis* (ATCC 35983). For lots with titers between 200 and 300 units/ml, a linear correlation of titers with their opsonic activity can be established (correlation coefficient 0.97, $P < 0.001$).

3.4. Opsonic activity

The opsonic activity of rabbit anti-20-kDa PS and anti-slime antibodies against the slime-producing *S. epidermidis* strain (ATCC 35983) was tested with a phagocytosis assay. Rabbit anti-slime antibodies promoted phagocytosis of *S. epidermidis* by $51 \pm 4\%$ (mean of five separate experiments in triplicate), whereas anti-20-kDa PS antibodies exhibited higher opsonic activity ($61 \pm 6\%$). Normal rabbit serum showed no opsonophagocytic activity and no cytotoxic effect. Consequently, antibodies to 20-kDa PS were able to opsonize and promote clearance of slime-producing *S. epidermidis*.

Eleven Sandoglobulin® lots were also examined for opsonic activity against *S. epidermidis* under the same experimental conditions. In all cases examined, Sandoglobulin® lots contained functional antibodies that promoted phagocytosis of bacterial cells by human polymorphonuclear leukocytes to an extent ranging from 35 to 75% (Fig. 6). The opsonic activity of Sandoglobulin® lots correlated well with the titers of 20-kDa PS specific antibodies. The opsonophagocytic effect of lots with titers lower than 200 units/ml varied from 30 to 50%, whereas lots with titers higher than 300 units/ml showed opsonic activity close to 75%. In the range of 200–300 units/ml, the content of 20-kDa PS specific IgG in Sandoglobulin® was linearly related ($r = 0.98$, $P < 0.001$) to *S. epidermidis* phagocytosis. These results demonstrate that the opsonic activity of the IVIG lots against slime-producing *S. epidermidis* may in part be attributed to the presence of 20-kDa PS specific antibodies in the preparations. Therefore, using the developed immunoassay one can determine the titers of specific anti-20-kDa PS IgG in IVIG lots that may be used as indicative markers of the opsonic activity.

3.5. Evaluation of Sandoglobulin® prophylactic efficacy in preterm neonates

In order to further correlate the estimated titers and the opsonic activity with the in vivo efficacy of Sandoglobulin®, a clinical trial with preterm neonates was performed. Preterm neonates re-

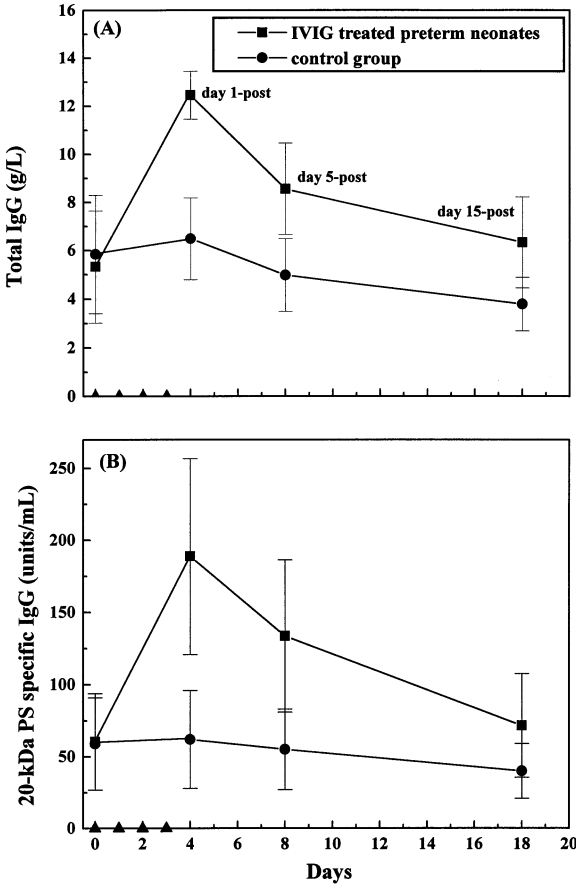

Fig. 7. Total IgG (A) and 20-kDa PS-specific IgG (B) in blood sera of low weight, preterm neonates who received prophylactically Sandoglobulin® (■) for 4 consequent days starting on the first day of their life. Blood sera were collected on day 0 (before IVIG infusion), and on days 1-, 5- and 15-post. Same parameters were determined in preterm neonates (●) who did not receive IVIG. Triangles (▲) on the horizontal axis show the days of IVIG infusion.

ceived Sandoglobulin® immediately after birth for 4 consequent days. Three Sandoglobulin® lots were used. No adverse reactions to the infusion were noted during the study. Total IgG concentrations were determined on days 0-, 1-, 5- and 15-post in blood sera of control and IVIG group. As shown in Fig. 7(A), on day 1-post, the IgG content was twice higher (level of significance, $P < 0.001$) than pre-infusion levels. In the following days, IgG levels followed a declining pattern. On day 5-post, IgG concentration (9.08 ± 2.19 g/l) was still significantly ($P < 0.05$) higher, while on day 15-post IgG concentration (6.19 ± 1.99 g/l) was comparable to pre-infusion levels (5.33 ± 2.35 g/l). These results indicated that the half-life time of immunoglobulin G in preterm neonates was approximately 15 days. At the same period, total IgG content in the control group was approximately 5 g/l and a considerable decline was observed on day 15-post.

Administrated Sandoglobulin® lots contained titers of 20-kDa PS specific antibodies higher than 200 units/ml, and had opsonic activity higher than 50%. 20-kDa PS specific IgG was measured in blood sera of all neonates (Fig. 7(B)). Following Sandoglobulin® infusion, the titers on day 1-post (188.9 ± 71.6 units/ml) and day 5-post (133.54 ± 52.7 units/ml) were significantly ($P < 0.01$) higher than the pre-infusion titer (60.36 ± 33.501 units/ml) and the respective values in the control group. On day 15-post, the antibody titers (71.513 ± 36.06 units/ml) to 20-kDa PS were comparable to those before Sandoglobulin® administration and higher than those in the control group.

From the clinical point of view, five preterm neonates out of 26 (19.2%) in the control group developed slime-producing *S. epidermidis* bacteremia, while in the IVIG treated group only one out of 26 (3.8%) developed slime-producing *S. epidermidis* bacteremia immediately after the last infusion. It should be noted, however, that 20-kDa PS specific IgG in this particular neonate before IVIG infusion was 2.72 units/ml, 22-times lower than the average value (60.36 units/ml) recorded for the neonates examined. These results demonstrated that administration of Sandoglobulin® containing titers of 20-kDa PS specific antibodies higher than 200 units/ml, and respective opsonic activities varying from 50 to 75%, seems to represent successful prophylaxis of preterm neonates against slime-producing *S. epidermidis* bacteremia.

4. Discussion

Intravenous immunoglobulin preparations constitute pooled normal intact polyspecific IgG obtained from several thousand healthy donors.

They are produced by a process of multiple alcohol precipitation, centrifugation, enzymic and/or chemical treatments, depending on the manufacturer [20]. The polyvalent immunoglobulin preparation used in the present study was Sandoglobulin®, which is a 7S intact IgG derived from plasma of more than 16 000 healthy donors from Central Europe. It was found to contain almost exclusively IgG, whereas IgM and IgA are present in minute amounts.

Intravenous immunoglobulin therapy has been established as routine treatment of immune deficiencies and other autoimmune and systemic inflammatory diseases. Its effectiveness, however, in prevention/treatment of infection in preterm neonates remains controversial despite the great number of clinical trials [14,15]. IVIG exert anti-infective function in neonates primarily through passive administration of opsonic antibodies against neonatal pathogens. It seems to facilitate inhibition of microbial attachment, phagocytosis and killing of bacteria by the macrophages. *S. epidermidis* has been identified as the commonest agent responsible for late-onset neonatal sepsis [2–6]. Slime-producing *S. epidermidis* bacteremia can be life threatening, since treatment with different antimicrobials is quite difficult [3–5,7]. Complementary treatment with IVIG is often recommended in these cases. Taking into account that 20-kDa PS is the major polysaccharide component and antigenic determinant of *S. epidermidis* slime, we developed an enzyme immunoassay in order to monitor specific antibodies against this polysaccharide in a significant number of IVIG preparations, as well as in blood sera of control and study group.

The titers of 20-kDa PS specific antibodies in various bottles of the same Sandoglobulin® lot were stable and, therefore, only titers in different preparation lots were evaluated. The majority of Sandoglobulin® lots contained relatively high amounts of 20-kDa PS-specific IgG. This fact further supports previous findings [13] demonstrating that the 20-kDa PS is highly immunogenic in humans probably due to the uniqueness of its structure. The calculated specific antibody titers ranged from 113.8 to 465.24 units/ml and were variable from lot-to-lot. This variability has been also reported by other investigators and has been associated with the conflicting results of clinical trials [16,17,20,21].

The opsonic activity of 11 Sandoglobulin® lots towards slime-producing *S. epidermidis* was evaluated. All lots promoted phagocytosis of slime-producing *S. epidermidis* by 35–75%. Weisman et al. [22] have also reported on the presence of opsonic *S. epidermidis* antibodies in IVIG preparations, which showed activity of high variability varying from very low to more than 90% in some lots. In the present report, where anti-slime sera and specific antibodies to the major slime antigenic determinant (20-kDa PS) were used, it is clearly shown that these specific antibodies are closely related with the Sandoglobulin® opsonic activity against slime-producing *S. epidermidis*. These differences may be attributed to the different donor pools. In our study Central Europe-derived IVIG preparations were used, whereas Weisman et al. [22] used preparations of USA volunteers. The opsonophagocytosis assay of rabbit anti-20 kDa PS and anti-slime antibodies on slime-producing *S. epidermidis* showed that both of them mediated significant phagocytosis of bacterial cells. The 20-kDa PS specific antibodies showed higher opsonic activity than anti-slime antibodies. This may be attributed to the way that the distinct macromolecules of slime are organized in space. Perhaps, the 20-kDa PS is localized in such a way that antibodies have easier access to it than other slime components. Since 20-kDa PS specific antibodies had significant opsonophagocytic effect, possible correlation between IVIG titers of 20-kDa PS specific IgG and opsonic activity was examined.

A sigmoid pattern correlation between IVIG antibody titers against the 20-kDa PS and the IVIG opsonic activity against slime-producing *S. epidermidis* was recorded. All lots with titers above 200 units/ml (75%) promoted phagocytosis of slime-producing *S. epidermidis* by 50–75%. In the range of 200–300 units/ml, this correlation was linear suggesting that quantification of specific antibody titers by the suggested immunoassay may allow prediction of the opsonic activity of the IVIG preparation. Hiemstra et al. [23] have also reported on such correlations for other bacterial strains. Summing up the in vitro results of the present

study, the majority of Sandoglobulin® lots (75%) contain specific, functional and opsonically active antibodies against 20-kDa PS.

IgG deficiency in preterm neonates accounts for increased susceptibility of preterm neonates to systemic infections. This is due to the fact that transplacental IgG transference occurs exclusively during the last trimester of pregnancy [24]. Furthermore, there is evidence that transplacental IgG antibodies to coagulase-negative staphylococci present in newborn serum are opsonically deficient [25]. IVIG administration to preterm neonates showed a concentration-dependent enhancing effect on opsonophagocytosis of coagulase-negative staphylococci [26]. Moreover, high titered IVIG lots significantly enhanced survival of animals with *S. epidermidis* sepsis when administered as a prophylactic agent [16]. Thus, the prophylactic potential of IVIG in preterm neonates against slime-producing *S. epidermidis* sepsis was evaluated. Sandoglobulin® was administrated for 4 consequent days immediately after birth to low birth weight (< 1700 g) infants. The administrated lots had anti-20-kDa PS IgG titers higher than 200 units/ml. Only one out of 26 (3.8%) IVIG treated neonates developed slime-producing *S. epidermidis* bacteremia, while five preterm neonates out of 26 (19.2%) in the control group developed slime-producing *S. epidermidis* bacteremia. A quite high degree of prophylaxis against slime-producing *S. epidermidis* bacteremia is suggested by these results.

Time course screening of total and specific IgG levels to the 20-kDa PS in neonatal blood sera revealed a significant increase of both titers at the period of 10 days after last infusion. The low levels of total and specific IgG recorded on day 0 (before infusion) are indicative of the fact that IgG transference from mother to fetus had not been completed in these preterm infants. The short half-life time of IgG in neonates is intriguing and may be ascribed to the IgG protection receptor. IgG protection receptor seems to be less abundant in neonates, i.e. more quickly saturated, resulting in increased catabolism of infused IgG molecules [27,28]. Another dose of Sandoglobulin® on day 8- or 9-post may be necessary to keep total and specific IgG levels elevated for a longer period. Neonates that developed slime-producing *S. epidermidis* septicemia had very low pre-infection 20-kDa PS specific IgG as compared to the average value, a fact that may constitute a factor contributing to the development of bacteremia.

Obtained results suggest that the developed enzyme immunoassay may well be used for evaluating IVIG content of specific antibodies against slime-producing *S. epidermidis*. Lots with titers higher than a cut-off value of 200 units/ml seem to provide adequate amounts of functional antibodies. Administration of such lots resulted in enhancement of host defense humoral responses and confers significant protection of premature neonates against slime-producing *S. epidermidis* bacteremia.

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