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Potential Use of Poly-*N*-Acetyl-β-(1,6)-Glucosamine as an Antigen for Diagnosis of Staphylococcal Orthopedic-Prosthesis-Related Infections▽

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Staphylococcus aureus **and coagulase-negative staphylococci are microorganisms most frequently isolated from orthopedic-implant-associated infections. Their capacity to maintain these infections is thought to be related to their ability to form adherent biofilms. Poly-*N*-acetyl-β-(1,6)-glucosamine (PNAG) is an important constituent of the extracellular biofilm matrix of staphylococci. In the present study, we explored the possibility of using PNAG as an antigen for detecting antibodies in the blood sera of patients with staphylococcal orthopedic-prosthesis-associated infections. First, we tested the presence of anti-PNAG antibodies in an animal model, in the blood sera of guinea pigs that developed an implant-associated infection caused by biofilm-forming, PNAG-producing strains of *Staphylococcus epidermidis*. Animals infected with *S. epidermidis* RP62A showed levels of anti-PNAG immunoglobulin G (IgG) significantly higher than those of the control group. The comparative study of healthy individuals and patients with staphylococcal prosthesis-related infections showed that (i) relatively high levels of anti-PNAG IgG were present in the blood sera of the healthy control group, (ii) the corresponding levels in the infected patients were slightly but not significantly higher, and (iii) only 1 of 10 patients had a level of anti-PNAG IgM significantly higher than that of the control group. In conclusion, the encouraging results obtained in the animal study could not be readily applied for the diagnosis of staphylococcal orthopedic-prosthesis-related infections in humans, and PNAG does not seem to be an appropriate antigen for this purpose. Further studies are necessary to determine whether the developed enzyme-linked immunosorbent assay method could serve as a complementary test in the individual follow-up treatment of such infections caused by PNAG-producing staphylococci.**

Staphylococcus epidermidis is mainly a normal inhabitant of healthy human skin and mucosal microflora, and as a commensal bacterium, it has a low pathogenic potential. In recent decades, however, *S. epidermidis* and other coagulase-negative staphylococci (CoNS) have emerged as a common cause of numerous nosocomial infections, mostly occurring in association with the use of medical devices, such as pacemaker electrodes, synthetic vascular drafts, urinary tract catheters, and orthopedic implants (29). It is thought that the pathogenicity of *S. epidermidis* is mostly due to its ability to colonize indwelling polymeric devices and form a thick adherent biofilm. Biofilms are often the cause for the difficulty in eradicating *S. epidermidis* bacteria on an indwelling device, since they provide significant resistance to antibiotics and to components of innate host defenses (26). Often, the removal and reinsertion of the device becomes necessary (27).

The early diagnosis of medical-device-related infections by the classical tools of microbiological analyses is difficult. The diagnosis is often made at advanced stages of infection, when often severe complications, such as the formation of abscesses, pain, and unsealing of the prosthetic devices, occur. Specific and noninvasive laboratory tests to diagnose these infections are not yet available. The detection of specific "antibiofilm" antibodies in the blood serum of patients could serve as a convenient noninvasive and inexpensive tool for the diagnosis of such foreign-body-associated infections.

Recently, Selan et al. described an enzyme-linked immunosorbent assay (ELISA) method to detect serum antibodies to staphylococcal slime-producing antigens, which gave promising results for the diagnosis of late-onset infections of synthetic vascular grafts (24). Staphylococcal slime-producing antigens, prepared from a patented slime-producing clinical isolate of *S. epidermidis*, are a mixture of polysaccharides which were not characterized chemically (M. Artini, F. Poggiali, G. Scoarughi, A. Cellini, F. Centofanti, and L. Selan, presented at the 12th International Symposium on Staphylococci and Staphylococcal Infections, Maastricht, The Netherlands, 3 to 6 September 2006).

Poly-*N*-acetyl-β-(1,6)-glucosamine (PNAG; also referred to as polysaccharide intercellular adhesin [PIA]) remains the most studied and well-characterized component of staphylococcal biofilm (17). It was shown to be immunogenic in mice (18) and to elicit protective immunity against both CoNS and *S. aureus*, suggesting its potential as a broadly protective staphylococcal vaccine (19).

A part of the *N*-acetylglucosamine residues in the PNAG

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backbone are de-N-acetylated, the degree of de-N-acetylation varying from one strain to another. In some strains, it is also partially replaced by *O*-succinyl groups (11, 17, 22, 23). It was recently shown that the nonacetylated epitopes of PNAG were the dominant targets of the human antibody response to staphylococcal PNAG (13). The comparison of two vaccines, one containing PNAG and the other containing its deacetylated derivative (dPNAG), conjugated to a carrier protein, showed that the dPNAG-conjugated vaccine was more effective in generating opsonic and protective antibodies. It was clearly shown that dPNAG contained epitopes against which opsonic killing antibody was directed (20).

For the elaboration of a diagnostic tool, we tested in this study the presence of anti-PNAG and anti-dPNAG antibodies in the blood sera of guinea pigs in an animal model which mimics an implanted-device-related infection caused by biofilm-forming, PNAG-producing strains of *S. epidermidis*. We also assessed the levels of antibodies against PNAG and dPNAG (immunoglobulin G [IgG] and IgM) in the blood sera of patients with staphylococcal infections of orthopedic prostheses (artificial hip and knee joints and fracture-fixation devices) and compared them with these levels in blood sera of healthy persons.

MATERIALS AND METHODS

Purification and chemical modification of PNAG. PNAG was prepared from the crude biofilm extract of *S. epidermidis* strain 5 (CIP 109562) of our collection (4) by gel permeation chromatography on a Sephadex S-300 column as described earlier (22). Fractions corresponding to PNAG were pooled and diluted with 100 mM sodium phosphate buffer, pH 7.4, to a final buffer concentration of 40 mM. The solution was filter sterilized, and the concentration of PNAG was determined by Morgan-Elson assay (5). PNAG was further diluted in 40 mM sodium phosphate buffer for the coating of the ELISA plates. To prepare dPNAG, 4 mg of the PNAG was dissolved in 1 ml of 4 M KOH in a screw-cap vial. Twenty milligrams of $NaBH_4$ was added to the mixture, and the vial was filled with nitrogen, heated at 95°C for 1 h, cooled, neutralized with 1 N HCl, dialyzed against deionized water, and lyophilized. We obtained nearly 100% deacetylation, judging from the colorimetric reactions and the ^{1}H-nuclear magnetic resonance (NMR) spectrum (data not shown). Milder conditions, described in the literature for the de-N-acetylation of PNAG from *S. aureus* MN8m (11), lead to an incomplete de-N-acetylation (15% of residual acetylation) (20).

The fully de-N-acetylated PNAG (0.5 mg) was dissolved in 50 μl of 5 M HCl (18) and immediately diluted in 40 mM sodium phosphate buffer to a final concentration of 1 μg ml^{-1}.The purity of PNAG and dPNAG was checked by ^{1}H NMR. NMR spectra were recorded at 25 and 30°C in D_2O on a Varian Unity Inova 500 instrument.

O polysaccharide from *Proteus mirabilis* strain G1 was a generous gift of E. Vinogradov (Institute for Biological Sciences, National Research Council, Ottawa, Ontario, Canada).

Guinea pig serum samples. We studied a humoral immune response to a staphylococcal biofilm-related infection by using a tissue cage (TC) animal model, developed earlier by our group (3). Briefly, a small multiperforated Teflon tube (i.e., the TC), filled with polymethylmethocrylat or titanium beads, was implanted subcutaneously in a flank of a guinea pig. A blood sample was taken from an ear of the animal prior to inoculation (control serum sample). Seven days after TC implantation, it was inoculated with a minimum infectious dose of a *S. epidermidis* strain (10^3 CFU). In order to verify whether the PNAG present in the biofilm was able to engender the production of specific antibodies in the serum of the host, the only strains used in this study were strong PNAG and biofilm producers in vitro. We therefore chose two strains, model strain *S. epidermidis* RP62A and a PNAG-producing strain from our collection, *S. epidermidis* 444 (CIP 109563) (22).

Two weeks after inoculation, the TCs were aseptically removed from the sacrificed animals, washed with saline, and sonicated in order to detach bacteria from the biofilm of the implants. A blood sample was taken from the each of the animals before euthanasia. EDTA was added to the blood samples to a final concentration of 0.2%, and serum was obtained after centrifugation at6,000 × *g* for 10 min at 20°C. Serum samples were aliquoted and stored at −80°C. Quantitative cultures were performed before and after washes and before and after the sonication in order to detect and enumerate bacteria attached to the implant as well as to confirm the identities and the purities of isolates. An animal having at least one infected implant (≥2.5 × 10^3 CFU/implant) was considered infected, and therefore, the serum of the blood sample taken before euthanasia was used as a positive sample. The negative control corresponded to the sera recovered from animals before TC implantation and from the noninfected animals.

Human serum samples. Sera were prepared from blood samples of patients who underwent an operation for the removal of an infected orthopedic prosthesis in Institut Calot (Berck-sur-mer, France), CHRU de Lille, and Centre Hospitalier Docteur Duchenne (Boulogne-sur-mer), collected between 2002 and 2007. The removed infected prostheses were analyzed for the presence of biofilm-related bacteria by a methodology developed in our laboratory. Briefly, freshly removed (within 24 h of surgery) prostheses (artificial hip and knee joints and different internal fixation devices, such as fixation pins, plates, and screws) were aseptically washed twice with saline and then sonicated in TSB. The microorganisms detached by sonication were considered biofilm related. Species identification was performed by using Gram stain morphology, a catalase test, the respiratory pattern, a coagulase test, and the ID ApiStaph system (bioMérieux, Marcy l'Etoile, France). In all cases, several different bacterial strains from each infected patient were identified. Staphylococcal isolates were screened for the presence of the *ica* locus as described earlier (4). Serum samples of patients with prostheses infected with at least one *ica*$^+$ strain were used in this study (10 blood samples). The following *Staphylococcus* species were identified: *S. epidermidis*, *S. capitis*, *S. hominis*, and *S. aureus*. Two of the patients were found to be infected with only *S. aureus*. Sera of 40 healthy volunteers (Etablissement Fraçais du Sang Nord de France) were used as negative controls.

ELISA. Microlon 600 plates (Greiner-Bio) were coated with 100 μl of PNAG or dPNAG at 1 μg ml^{-1} in 40 mM sodium phosphate buffer overnight at room temperature. The plates were rinsed three times with phosphate buffer and blocked with 5% skim milk in Tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.3, 150 mM NaCl) for 2 h. Sera diluted in TBS–0.05% Tween 20 were then added, and plates were incubated for 1 h, washed six times with TBS-Tween 20, and incubated for 1 h with appropriate secondary antibody, diluted 1:2,000 in TBS-Tween 20. The plates were then washed three times with TBS-Tween 20, twice with TBS and once with the appropriate color development buffer. For guinea pig sera, alkaline phosphatase-conjugated sheep anti-guinea pig IgG (Biovaley, Conches, France) was used. The color was developed with 100 μl of 15 mM *p*-nitrophenyl phosphate in alkaline phosphatase buffer (1 M diethanolamine, pH 9.8, with 0.5 mM $MgCl_2$) for 30 min in the dark, according to the manufacturer's instructions. The optical density (OD) at 405 nm was measured using a μQuant microplate reader (BioTek Instruments, Winooski, VT). For human sera, horseradish peroxidase-conjugated rabbit anti-human IgG (Sigma) or goat anti-human IgM (Sigma) was used, and the color was developed with 100 μl of a substrate solution (0.1% *O*-phenylene diamine [Sigma] in 50 mM phosphate-citrate buffer, pH 5.0, activated with 0.8 μl of 30% H_2O_2 per ml) for 15 min in the dark, and the OD at 490 nm was measured. Background readings corresponding to the control wells incubated with blocking solution not containing sera were subtracted automatically. For each serum dilution, readings corresponding to the nonspecific binding (control wells filled with phosphate buffer not containing PNAG) were subtracted. Each experiment was performed at least twice in triplicate. Antibody titers are expressed as units of OD.

A competition ELISA was performed essentially as described by Kelly-Quintos et al. (13). Briefly, ELISA plates were sensitized with PNAG or dPNAG and blocked as described above. Serum samples to be analyzed were diluted to a concentration twice that which would result in OD readings of ~1.0. Dilutions (0.25 to 4 μg ml^{-1}) of PNAG or the O polysaccharide from *Proteus mirabilis* strain G1 (25) were mixed with an equal volume of the diluted serum samples and incubated for 30 min, and the mixture was added to the antigen-coated and blocked well. The remainder of the ELISA continued as described above.

Statistical analysis was performed using a Mann-Whitney U test (http://elegans.swmed.edu/~leon/stats/utest.html).

RESULTS

Determination of optimum working conditions for ELISA. After assaying concentrations ranging from 0.001 to 10 μg ml^{-1} for coating the ELISA plates, we chose 1 μg ml^{-1} as an optimal concentration for both PNAG and dPNAG. Assays

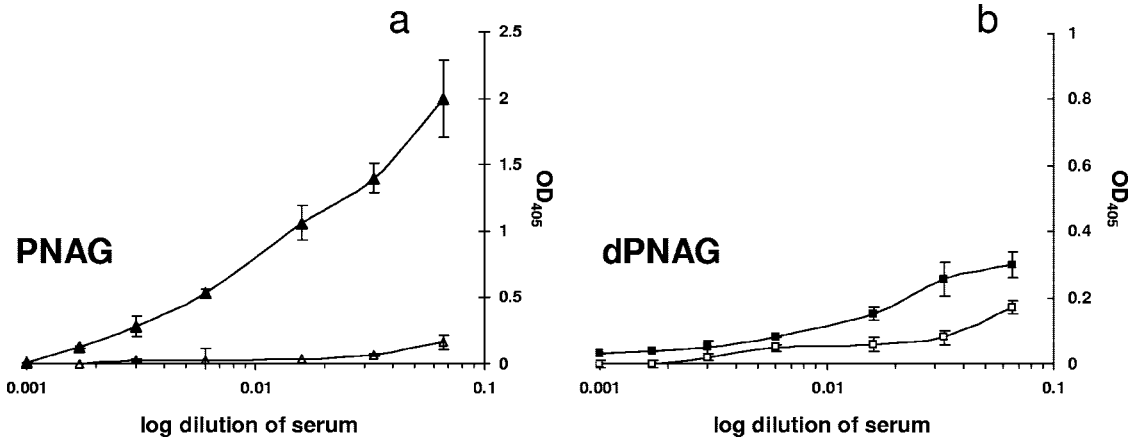

FIG. 1. Detection of IgG antibodies to PNAG (a) and dPNAG (b) in pooled sera collected from nine guinea pigs prior to the implantation of the TC (open triangles and squares) and five guinea pigs which developed an implant-related infection with *S. epidermidis* RP62A (filled triangles and squares). Serum samples were diluted at 1:1,000, 1:600, 1:300, 1:150, 1:60, 1:30, and 1:15.

with guinea pig and human sera gave similar results (data not shown).

Serial dilutions of sera from guinea pigs which developed an implant-related staphylococcal infection and the sera of noninfected animals were tested by ELISA. Figure 1 shows data for the detection of antibodies to PNAG and dPNAG in the pooled sera collected from nine guinea pigs prior to the implantation of the TCs and five guinea pigs which developed an implant-related infection with *S. epidermidis* RP62A. It is clear that titers of antibody to PNAG in the pooled sera of the infected animals were significantly higher than those of the control animals (Fig. 1a). The level of antibodies to dPNAG was lower, and the difference between the healthy and infected animals was less pronounced (Fig. 1b). Therefore, for the consecutive studies of the individual responses, we used only PNAG as the coating agent. The dilutions of guinea pig sera were chosen to be 1:30, corresponding to the linear range of the curve with the highest inclination (Fig. 1a), as described by Kolonitsiou et al. (15).

Determination of specificity of antibodies in guinea pigs. The specificity of antibodies for PNAG in sera of infected guinea pigs was studied by competition ELISA. Separate incubation of sera with increasing concentrations of PNAG reduced the amount of antibodies in the mixture that could interact with the antigen on the microplate and, therefore, the recorded absorbance. The results were expressed as percentages of the inhibition of binding. The O polysaccharide from *Proteus mirabilis* strain G1, a high-molecular-weight polysaccharide containing both positively charged L-lysine (L-Lys) and negatively charged glucoronic acid (GlcA) groups (25) at the same concentrations as PNAG was used as a control in order to exclude the possibility of nonspecific binding. Figure 2 shows a pattern of inhibition of binding of IgG antibodies to PNAG in the sera of guinea pigs infected with *S. epidermidis* RP62A. The addition of PNAG at 2 μg ml^{-1} to the pooled sera of guinea pigs infected with *S. epidermidis* RP62A led to an almost complete inhibition of binding. At the same time, the addition of O polysaccharide from *P. mirabilis* G1 at up to 4 μg ml^{-1} did not have any effect on binding (data not shown). These data confirm that the interaction of sera of infected guinea pigs with the PNAG was due to the presence of the specific anti-PNAG antibodies.

Individual antibody response in infected guinea pigs. We measured titers of antibodies against PNAG in sera of 9 control guinea pigs (group A) and 17 guinea pigs with implant-associated infection (5 infected with *S. epidermidis* RP62A [group B] and 12 infected with *S. epidermidis* 444 [group C]). Figure 3 shows that on average, the titers of IgG antibodies against PNAG in guinea pigs infected with *S. epidermidis* RP62A were significantly higher than those in guinea pigs from the control group ($P < 0.01$). However, levels of antibodies in guinea pigs infected with *S. epidermidis* RP62A were highly variable, and certain overlap between the values of infected and noninfected guinea pigs cannot be neglected. Furthermore, there were no significant differences (P value, ~0.1) between the levels of antibodies in control guinea pigs and those in animals infected with *S. epidermidis* 444 (Fig. 3).

We followed the evolution of the antibody response to PNAG in individual guinea pigs after their implant-related

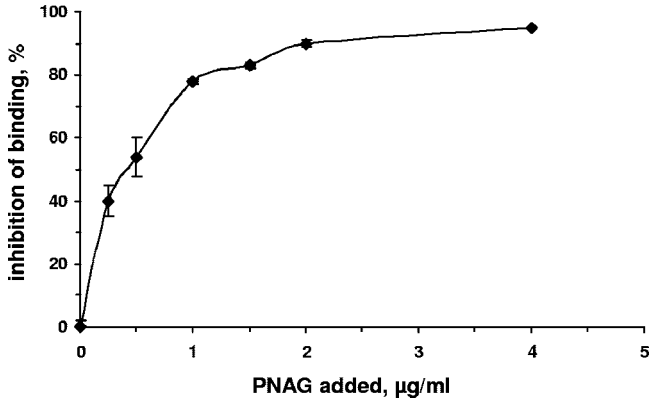

FIG. 2. Competition ELISA results demonstrating the inhibition of binding of antibodies to PNAG in the sera of infected guinea pigs. Binding of the pooled sera collected from guinea pigs which developed an implant-related infection with *S. epidermidis* RP62A to a PNAG-coated plate was inhibited by using various concentrations of PNAG (0.25, 0.5, 1, 1.5, 2, and 4 μg ml^{-1}).

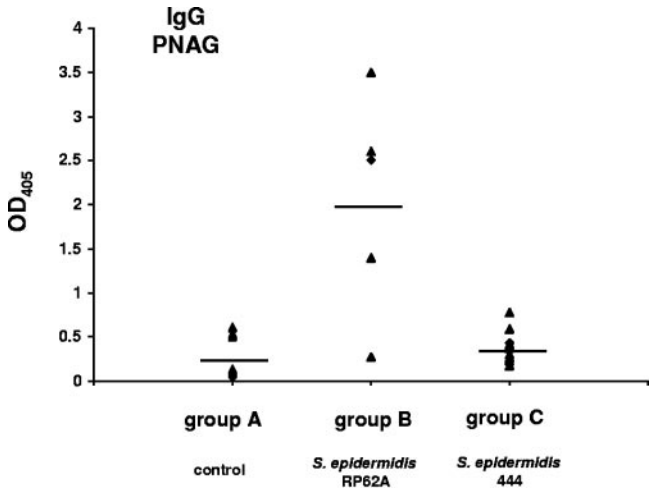

FIG. 3. Detection of IgG antibodies to PNAG in sera of control guinea pigs (group A) and guinea pigs with implant-associated infections with *S. epidermidis* RP62A (group B) and *S. epidermidis* 444 (group C). IgG titers in animals of group B were significantly higher than those of the control group ($P < 0.01$). There was no significant difference (P value, ~0.1) between the levels of antibodies in the control group and group C. Average values are indicated by horizontal lines.

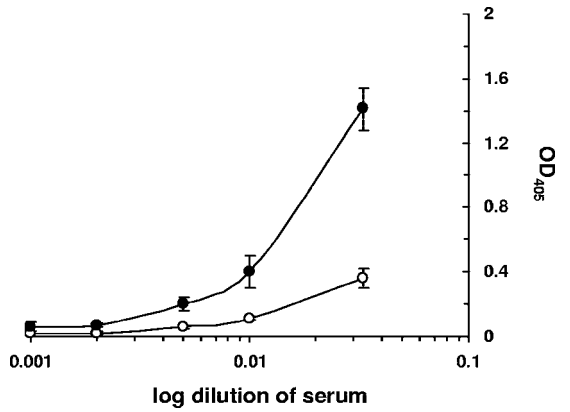

FIG. 4. Detection of IgG antibodies to PNAG in blood serum of a guinea pig before (open circles) and after (filled circles) an implant-associated infection with *S. epidermidis* 444. Serum was diluted at 1:1,000, 1:600, 1:200, 1:100, and 1:30.

infection with PNAG-producing *S. epidermidis*. Figure 4 shows a typical pattern of titers of IgG antibodies to PNAG in a guinea pig before (panel a) and after (panel b) an implant-associated infection by *S. epidermidis* 444. Sera from a total of nine guinea pigs before and after the infection were tested. Seven guinea pigs showed a higher level of IgG antibodies to PNAG after the infection.

Presence of the PNAG- and dPNAG-specific antibodies in patients' sera. Plates were coated with PNAG and dPNAG at optimal concentrations (1 μg ml^{-1}) in phosphate buffer. Blood sera of 40 healthy individuals were grouped into four pools with 10 samples of blood serum in each pool (group 1). Blood serum from each patient with a diagnosed biofilm-related staphylococcal infection (10 samples total) was tested individually (group 2). The determination of optimum dilutions of blood sera was performed by incubating the coated microplates with serial dilutions of sera. The corresponding absorbance values were plotted in semilog coordinates, as for the guinea pig sera (see above). The chosen optimum dilutions were 1:500 for anti-PNAG IgG, 1:200 for anti-dPNAG IgG, and 1:10 for anti-PNAG and anti-dPNAG IgM.

Screening of negative control pools and patient sera revealed the relatively high levels of anti-PNAG IgG in the sera of healthy individuals. The level of this IgG in patient sera was very variable and overall higher than that in control sera, but the difference was statistically insignificant ($P > 0.05$) (Fig. 5a).

The levels of anti-dPNAG IgG in healthy individuals were lower than those of anti-PNAG IgG, but again, the overall difference between the healthy and infected subjects was insignificant ($P > 0.05$) (Fig. 5b). We performed a competition ELISA experiment in order to confirm the specificity of antibodies in samples of both groups. Figure 6 shows the inhibition of binding of IgG to PNAG in the sera of a control pool of sera from 10 healthy subjects and the serum of a patient diagnosed with *S. epidermidis* prosthesis-related infection. The addition of PNAG at 2 μg ml^{-1} to both samples led to a significant inhibition of binding (90% for the patient serum and 80 to 85% for the control pools). The addition of O polysaccharide from *P. mirabilis* G1 at the same concentrations did not have any effect on binding (data not shown). These data confirm the specificity of the antibodies in healthy individuals and in patients.

The levels of anti-PNAG and anti-dPNAG IgM were low in both groups, except for one case in group 2 (Fig. 7). For the patient in question, two different *S. epidermidis* strains were found at the infected prosthesis. One of them seemed to be a PNAG producer, judging from the action of the PNAG-degrading enzyme dispersin B (12) on its extracellular matrix (data not shown).

DISCUSSION

To date, no efficient treatment of implant-associated infectious bacteria has been proposed. Their detection by classical tools of microbiological analysis is not sensitive enough. Often when cultures taken at the puncture of the infection site become positive, the infection is already at an advanced stage and removal of the prosthesis in order to increase the efficiency of the antibiotic therapy becomes unavoidable. There is a necessity to develop efficient tests allowing the diagnosis of such infections in order to improve medical decision making.

The discovery of PNAG (PIA), a specific component of staphylococcal biofilm, inspired hopes in terms of its potential therapeutic application against medical-implant-related infections. Its potential as a broadly protective vaccine for many clinically important staphylococci has been suggested (19). In our recent studies, we explored the possibility of using PNAG as a target for the enzymatic removal of staphylococcal biofilm (2). In the present study, we investigated another prospect for its potential application. We attempted to assess its utility as an antigen for the detection of prosthesis-associated infections.

Earlier publications suggested that the staphylococcal extracellular matrix "was very likely PIA" and in case of well-studied model strains of *S. epidermidis*, "PIA and slime are the same" (8). Later, evidence for alternative, PNAG-independent mech-

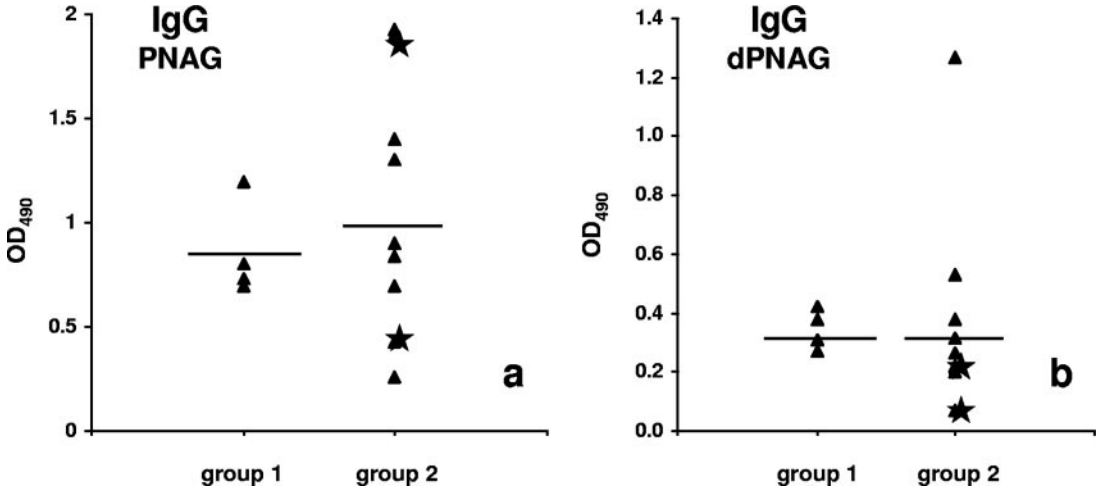

FIG. 5. Detection of IgG antibodies to PNAG (a) and dPNAG (b) in pooled sera collected from 40 healthy individuals (four pools with 10 samples of sera in each pool) (group 1) and 10 patients with prosthesis-related staphylococcal infections (group 2). Values corresponding to two patients infected with *S. aureus* are indicated with stars. The overall differences between the groups 1 and 2 were statistically insignificant ($P > 0.05$). Average values are indicated by horizontal lines.

anisms for staphylococcal biofilm formation was presented (6, 14, 21). According to different epidemiological studies, ~60% of *S. epidermidis* strains isolated from an orthopedic-implant-related infection carried the *ica* locus and were therefore able to produce PNAG in vivo (1). Around 75% of CoNS strains from our collection of staphylococci isolated from infected orthopedic prostheses are positive for *ica* (4). Since, PNAG and its de-N-acetylated derivative (dPNAG) were previously shown to be immunogenic in humans (13), it was a possibility that at least half the patients with orthopedic-prosthesis-related infection would develop specific anti-PNAG or anti-dPNAG antibodies. Detection of these antibodies in their blood sera could indicate the presence of a staphylococcal infection and could help, in conjunction with other tests, to diagnose the infection.

As a first step, we attempted to understand whether an implant-associated infection could cause the production of PNAG-specific antibodies in an animal model. Thanks to a recently developed guinea pig model mimicking an implant-related infection (3) and a collection of staphylococcal strains with a well-characterized biofilm composition (22, 23), we were able to study cases of infection caused by PNAG-producing staphylococcal strains.

Our results indicated that in the animal model of guinea pigs with implant-related infection caused by a PNAG-producing strain (*S. epidermidis* RP62A), animals were producing specific anti-PNAG IgG after two weeks of infection. The levels of specific anti-dPNAG IgG were also slightly higher in infected

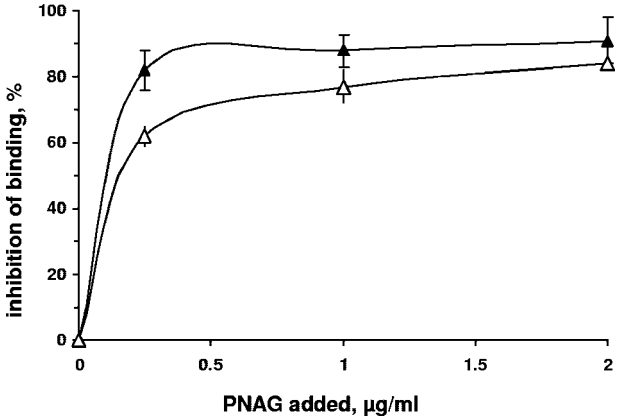

FIG. 6. Competition ELISA results demonstrating the inhibition of binding of antibodies to PNAG in the pooled sera of healthy individuals (open triangles) and a blood serum sample of a patient with a prosthesis-related *S. epidermidis* infection. Binding of the sera to a PNAG-coated plate was inhibited by using various concentrations of PNAG (0.25, 1, and 2 $\mu g\ ml^{-1}$).

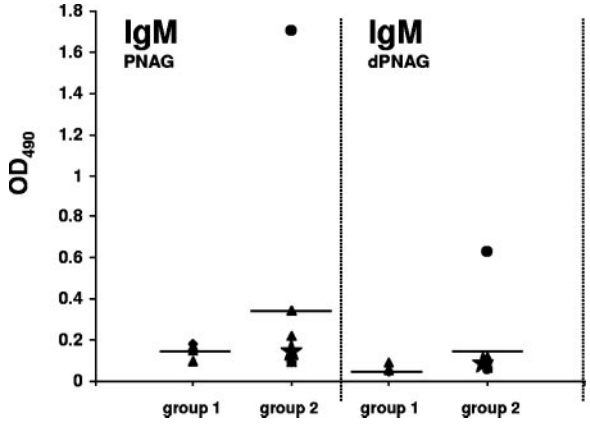

FIG. 7. Detection of IgM antibodies to PNAG and dPNAG in pooled sera collected from 40 healthy individuals (four pools with 10 samples of serum in each pool) (group 1) and 10 patients with staphylococcal prosthesis-related infections (group 2). Values corresponding to two patients infected with *S. aureus* are indicated with stars, and those of a patient having the greatest IgM response are indicated with circles. The overall differences between groups 1 and 2 were statistically insignificant ($P > 0.05$). Average values are indicated by horizontal lines.

animals than in control animals. These data support the assumptions that PNAG was produced in vivo and was accessible to the host humoral immune defense and that antibodies to acetylated and, to a lesser extent, deacetylated epitopes were produced. In the same model, *S. epidermidis* 444, a clinical strain from our collection, on average elicited a weaker antibody response. Knowing that this strain in vitro makes biofilms in which protein components are present in important amounts (2), we can speculate that in the in vivo biofilms, PNAG is less exposed to the immune system or its relative quantity is not always sufficient for the production of antibodies in the blood serum of the animal.

The analysis of human sera led to more ambiguous results. Screening of patient's sera and sera of healthy individuals revealed a relatively high level of anti-PNAG IgG in the sera of healthy controls. If this result is rather disappointing, it is nevertheless interesting to try to understand the reason for this phenomenon. Despite the fact that the presence of the *ica* operon is considered a marker discriminating between clinical-device-associated strains and skin flora (7, 16), an important percentage of commensal CoNS strains in healthy individuals are *ica* positive and potentially capable of producing PNAG. Another explanation could be provided by the recently published evidence that a variety of gram-negative pathogens, including *Escherichia coli*, *Actinobacillus actinomycetemcomitans*, *Actinobacillus pleuropneumonia*, *Bordetella pertussis*, *Burkholderia cepacia*, and *Yersinia pestis*, have a genetic locus *pga* coding for a polysaccharide structurally identical to PNAG (12) and therefore could produce an equivalent of PNAG. This was definitively shown for *E. coli* (28), *Actinobacillus pleuropneumoniae* (9), and human oral pathogen *A. actinomycetemcomitans* (10). The presence of anti-PNAG IgG in sera of healthy individuals could thus be explained by their natural exposure to PNAG-producing gram-negative bacteria, presence of these antigens in common vaccine preparations, and previous infections and nasal carriage of *S. aureus* (Tomas Maira-Litrán, personal communication).

We did not observe any important differences among the levels of anti-PNAG IgM and anti-dPNAG IgG and IgM in healthy subjects and patients with orthopedic-prosthesis-related infections caused by CoNS. Only one patient out of 10 showed an elevated level of anti-PNAG and anti-dPNAG IgM. Therefore, our results showed that dPNAG was not more suitable than PNAG for the immunological diagnosis of orthopedic-prosthesis-related infections.

To summarize, our results show that PNAG-producing strains seem to cause an increase in anti-PNAG IgG antibodies in the guinea pig model for implant-related infection. However, PNAG does not seem to be a convenient antigen for a diagnostic tool which could discriminate patients with implant-related staphylococcal infection and healthy individuals.

As a tool complementary to other tests and as a method for cases of infections with PNAG-producing staphylococci, the developed ELISA method can be helpful for a follow-up of patients after explanting the infected prosthesis and consecutive antibiotic treatment. In order to develop a reliable diagnostic tool for the diagnosis of medical-device-related infections, other antigens should be investigated.

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