Antibodies to Staphylococcal Peptidoglycan and Its Peptide Epitopes, Teichoic Acid, and Lipoteichoic Acid in Sera from Blood Donors and Patients with Staphylococcal Infections

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Antibodies to the staphylococcal antigens peptidoglycan, β-rhibitol teichoic acid, and lipoteichoic acid, as well as to the peptidoglycan epitopes L-Lys-D-Ala-D-Ala, L-Lys-D-Ala, and pentaglycine, were found over a wide range of concentrations in sera from both blood donors and patients with verified or suspected staphylococcal infections. The patient group was heterogeneous with regard to both age and type of staphylococcal infections, being representative for sera sent to our laboratory. In single-antigen assays antibodies to pentaglycine had the highest predictive positive value (67%), although only 32% of the patients had elevated levels of such antibodies. Combinations of test antigens could yield positive predictive values as high as 100%, but then the fraction of positive sera was low. Indeed, the fraction of patient sera which was positive in multiple-antigen tests never exceeded 61%. The clinical usefulness of these seroassays for identifying Staphylococcus aureus as a causative agent was limited, owing to the considerable overlap in the range of antibody concentrations between patient and blood donor sera.

Serodiagnosis of serious Staphylococcus aureus infections has been reported to be successful for selected groups of patients (7). A variety of staphylococcal antigens, including whole staphylococci (15), crude cell wall material (5), peptidoglycan (PG) (4, 27, 32), teichoic acid (27, 28, 33), lipoteichoic acid (LTA) (32), α-toxin (7, 27, 28), β-toxin (7), nucleos (27), and lipase (6), have been used. In addition, a synthetic peptide corresponding to the PG epitope L-Lys-D-Ala-D-Ala has been used (10, 35).

In this study we used the cell wall antigens PG, β-rhibitol teichoic acid (β-RTA), and LTA. The chemical structure and immunological properties of these antigens are well documented (8, 17, 18, 23, 25); neither is exclusive for staphylococci.

Bacterial PG, present in most bacteria, is composed of sugar chains with tri-, tetra-, or penta- tepeptides covalently linked to the sugar chains. These sugar-peptide chains are interlinked by short peptid bridges or directly through a diamin acid. The interpeptide bridge in S. aureus PG consists of five glycine residues and is considered to be specific for staphylococcal PG. Both the NH₂ and COOH termini of the peptid bridge can bind antibodies (13, 22, 24, 26).

Bacterial wall teichoic acids are polymers possessing phosphodiester polyls with or without sugar residues, occasionally also with d-alanine ester residues. The teichoic acid from S. aureus contains ribitol with N-acetylglicos- aminyl residues (3).

Membrane LTA consists of a glycerol teichoic acid linked at one end to a glycolipid. Staphylococcal LTA, like most others, is variously substituted with d-alanine ester residues and mono- or oligosaccharides (9). The cell wall and membrane teichoic acids are believed to be present in many gram-positive bacteria (30).

In this study we analyzed sera from blood donors and from a heterogeneous group of patients with verified or suspected staphylococcal infections for antibodies to purified and chemically characterized PG, β-RTA, and LTA and to the synthetic PG epitopes L-Lys-D-Ala-D-Ala, L-Lys-D-Ala, and pentaglycine (Gly₃).

MATERIALS AND METHODS

Antigens. PG and LTA were purified from S. aureus Cowan 1 NCTC 8530 as described by Park and Hancock (21) and Aasjord al. (2), respectively. β-RTA was isolated from S. aureus Wood 46 NCTC 10344 as described by Osland et al. (20). The chemical compositions of PG, LTA, and β-RTA were as described by Wergeland and Endresen (31), Aasjord and Grov (1), and Haukenes (12), respectively.

The synthetic peptides Gly₃ (Sigma Chemical Co.) and diacetyl-L-Lys-D-Ala-D-Ala and diacetyl-L-Lys-D-Ala (Bio- products) were covalently coupled to human albumin (Sigma) by N-succinimidyl 3-(2-pyridyldithio)propionate (Pharmacia, Uppsala, Sweden) in accordance with manufacturer instructions. Free thiol groups from the reduction of the 2-pyridyl disulfide groups introduced on albumin were determined prior to and after peptide couplings with ¹⁴C-iodoacetamide (New England Nuclear Corp., Boston, Mass.). The reduced number of free thiol groups per mole of albumin thus corresponded to moles of peptides introduced. Between 7 and 8 mol of peptide were coupled to each mole of albumin, for a coupling efficiency of more than 97%.

Sera. All serum samples were obtained from Haukeland Hospital, Bergen, Norway, and immediately stored in aliquots at −80°C. Patients were defined as having a verified staphylococcal infection if the clinical laboratory found significantly raised antistaphylococcal titers or if staphylococci could be isolated from clinical specimens. Two or more serum samples from patients with verified (n = 42) or

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suspected \((n = 11)\) staphylococcal infections and from patients with other bacterial infections \((n = 9)\) were collected. A total of 36 of the patients were between 14 and 78 years old (adult group), and 26 were under 14 years old (child group). The adult patients with verified staphylococcal infections \((n = 31)\) had osteomyelitis \((n = 16)\), septicemia \((n = 3)\), arthritis \((n = 4)\), endocarditis \((n = 2)\), and tissue infections \((n = 6)\). Three of the patients had suspected staphylococcal infections like osteomyelitis \((n = 2)\) and arthritis \((n = 1)\). One patient had endocarditis caused by gram-negative bacteria, and another had streptococcal bursitis. The children with verified staphylococcal infections \((n = 11)\) had osteomyelitis \((n = 7)\), arthritis \((n = 1)\), and tissue infections \((n = 3)\). Three of the eight children with suspected staphylococcal infections had osteomyelitis. Two had infections caused by meningococci, one had an infection caused by streptococci, and four had infections caused by gram-negative rods. Serum samples were also collected from 66 blood donors. Aliquots of serum samples from 25 random donors were pooled and used as a control serum.

**Antibody assay.** An enzyme-linked immunosorbent assay (ELISA) was done by standard procedures as described earlier \((31)\) with polystyrene enzyme immunoassay plates \((96\) wells; Costar, Cambridge, Mass.). Coating was performed with sonicated PG \((15 \text{ min at } 20 \text{ KHz})\) diluted in \(0.01\) M phosphate-buffered saline \((\text{pH } 7.2)\) to a concentration of \(5\) \(\mu\)g/ml. The coating concentrations of LTA and \(\beta\)-RTA were \(5\) and \(25\) \(\mu\)g/ml of phosphate-buffered saline, respectively. The optimal coating concentrations of albumin-L-Lys-D-Ala-D-Ala, albumin-L-Lys-D-Ala, and albumin-Gly\(_\text{S}\) were all found to be \(15\) \(\mu\)g/ml of phosphate-buffered saline. ELISA readings of optical densities (ODs) at 492 nm will be referred to as antibody values. The day-to-day variation was monitored by using the pooled control serum at the same working dilutions as the test sera. The nonspecific background values, obtained when phosphate-buffered saline containing \(0.05\%\) Tween 20 was added instead of a serum sample, resulted in ODs of <0.15 in all assays. Multiple serum samples from individual patients were always analyzed with all antigens in the same experimental set-up.

**Immunoglobulin concentrations.** Total immunoglobulin G (IgG), IgA, and IgM were routinely determined by the immunological laboratory for all blood donor and patient sera with a Nephelometer-Analyzer (Behring Institute). Normal ranges of immunoglobulin concentrations in adult sera were, according to a local laboratory standard, 7 to 18, 0.5 to 3.3, and 0.3 to 2.5 g/liter for IgG, IgA, and IgM, respectively. A local laboratory standard was also used for sera from children at various ages.

**Statistics.** Arithmetic means were used throughout. The correlation coefficients \((r)\) were calculated by the least-squares linear correlation method. Differences between groups were evaluated by a two-tailed Student \(t\) test.

**Predictive values.** Positive antibody values were defined as being greater than or equal to the mean plus one standard deviation (SD) of blood donor antibody values. We defined a positive predictive value \((\text{PV-P})\) as the percentage of patients with verified staphylococcal infections who were seropositive among all seropositives and a negative predictive value \((\text{PV-N})\) as the percentage of seronegative noninfected individuals among all seronegatives.

**RESULTS**

The optimal working dilutions of sera which best separated the antibody values of blood donors and patients were found to be \(1:2,000, 1:16,000, 1:1,000,\) and \(1:250\) for PG, \(\beta\)-RTA, LTA, and the albumin-peptide conjugates, respectively.

**Antibodies reactive with the staphylococcal antigens and the PG epitopes.** The ELISA values for the antibodies in blood donor and patient sera reactive with the various antigens are presented in Table 1. The results show wide ranges of antibody values, with the highest coefficients of variation occurring for antibodies to \(\beta\)-RTA and PG in both patients and blood donors (Table 1). Figure 1 shows the results of tests of individual patient sera with the various staphylococcal antigens. Samples of sera from patients with verified bacterial infections were taken at the time of laboratory diagnosis. In the case of sera from patients with suspected, but later not verified, bacterial infections, samples were collected at the time of tentative clinical diagnosis. Sera from adult patients (Fig. 1A) were considered positive if the OD exceeded the mean OD of the blood donor sera plus one SD. Less than 50% of these sera were positive in any of the tests, the fractions of positives being 42% (PG), 39% (L-Lys-D-Ala-D-Ala), 32% (L-Lys-D-Ala and Gly\(_\text{S}\)), 35% (LTA), and 29% (\(\beta\)-RTA). Sera from adult patients with verified nonstaphylococcal infections were generally negative. The exceptions were one patient with a gram-negative rod infection who was positive for antibodies to L-Lys-D-Ala and one patient with a beta-hemolytic streptococcus infection who was positive for antibodies to Gly\(_\text{S}\).

For all patients in this study at least two serum samples were available. Generally, no significant difference in antibody reactivity in serial samples could be detected. For example, 10 serum samples were collected from each of two patients with \(S.\ aureus\) osteomyelitis, the sampling periods being 2 years and 2 months, respectively. Neither of these serial samples showed any significant antibody fluctuations.

All sera from children contained antibodies reactive with the cell wall antigens and the PG epitopes (Fig. 1B), but antibody values were generally lower than in adults. Of the 26 children, 7 were 2 years old or younger, and sera from them contained low levels of IgG against the cell wall antigens and the Gly\(_{\text{S}}\) epitope but rather high levels of antibodies to the L-Lys-D-Ala-D-Ala and L-Lys-D-Ala epitopes. One 7-year-old child with streptococcal osteomyelitis possessed high levels of antibodies to PG and the L-Lys-D-Ala-D-Ala and L-Lys-D-Ala epitopes but low levels of antibodies to the other antigens. About 50% (16 of 31) of the adult patients had staphylococcal osteomyelitis. Six (38%) were positive for antibodies

| TABLE 1. Ranges of antibody values in sera from blood donors and patients with staphylococcal infections tested with various staphylococcal antigens |
|-----------------|-----------------|-----------------|
| **Antigen (working serum dilution)** | **Blood donors** | **Patients** |
|                 | Mean SD | Coefficient of variation (%) | Mean SD | Coefficient of variation (%) |
| LTA (1:1,000)   | 0.53 0.22 | 42  | 0.61 0.29 | 45  |
| \(\beta\)-RTA (1:16,000) | 0.79 0.45 | 57  | 0.90 0.54 | 54  |
| PG (1:2,000)    | 0.68 0.32 | 45  | 1.15 0.63 | 55  |
| L-Lys-D-Ala-D-Ala (1:250) | 0.62 0.24 | 37  | 0.85 0.32 | 37  |
| L-Lys-D-Ala (1:250) | 0.62 0.27 | 43  | 0.91 0.37 | 41  |
| Gly\(_{\text{S}}\) (1:250) | 0.41 0.21 | 37  | 0.60 0.29 | 37  |

*Mean and SD are given as ODs at 492 nm.*
to β-RTA, four (25%) were positive for antibodies to LTA, L-Lys-D-Ala-D-Ala, and L-Lys-D-Ala, and three (19%) were positive for antibodies to PG and Gly5.

**Immunoglobulin concentrations in serum.** All blood donors had serum immunoglobulin concentrations (IgG, IgA, and IgM) within the normal range. One adult patient with a verified staphylococcal infection had below-normal concentrations of IgG and IgA. On the other hand, 26, 52, and 32% had elevated concentrations of IgG, IgA, and IgM, respectively. Two of the children (8%) had low IgG concentrations, but none had low concentrations of IgM or IgA. Of 17 children with verified or suspected staphylococcal infections, 35% (n = 6), 53% (n = 9), and 33% (n = 9) had elevated concentrations of IgG, IgA, and IgM, respectively. All of the seven children less than 2 years old had elevated concentrations of IgM antibodies, and one of them also had elevated levels of IgG and IgA antibodies.

**Differences between antibody levels.** Analyses of differences between levels of antibodies to the various antigens in sera from adult patients and blood donors were done. Significant differences were found for PG (P < 0.001), L-Lys-D-Ala (P < 0.001), L-Lys-D-Ala-D-Ala (P < 0.01), and Gly5 (P < 0.05) but not for β-RTA and LTA antibody levels.

**Correlation coefficients.** Positive correlations with r values ranging from 0.484 to 0.976, were found between antibodies to the various antigens in sera from adult patients (Table 2). Sera from blood donors showed a significant correlation (P < 0.001) between antibodies to PG and LTA and also between antibodies to the three PG epitopes. Generally, all r values were higher in the patient group, the exception being comparisons between peptide epitopes, for which r values approached 1 in both groups.

We analyzed the correlation of total serum IgG with levels of antibodies to the various antigens. These analyses (r values not presented) showed that only antibodies to PG and LTA were significantly (P < 0.01) positively correlated with total IgG in adult patient sera, the r values being 0.486 and 0.458, respectively.

The low r values and fractions of positives in each test indicated that combining two antigens would increase the correlation of total serum IgG with antibodies to each of the antigens.

**TABLE 2. Coefficients of correlation between antibodies to various staphylococcal antigens in sera from blood donors and patients with staphylococcal infections**

<table>
<thead>
<tr>
<th>Antibodies correlated (antigens to)</th>
<th>Correlation coefficient (r) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood donors (n = 66)</td>
</tr>
<tr>
<td></td>
<td>Adult patients (n = 31)</td>
</tr>
<tr>
<td>PG and L-Lys-D-Ala</td>
<td>0.184*</td>
</tr>
<tr>
<td>PG and L-Lys-D-Ala-D-Ala</td>
<td>0.163*</td>
</tr>
<tr>
<td>PG and Gly5</td>
<td>0.183*</td>
</tr>
<tr>
<td>PG and β-RTA</td>
<td>0.945*</td>
</tr>
<tr>
<td>LTA and β-RTA</td>
<td>0.976*</td>
</tr>
<tr>
<td>L-Lys-D-Ala and Gly5</td>
<td>0.966*</td>
</tr>
<tr>
<td>L-Lys-D-Ala-D-Ala and Gly5</td>
<td>0.964*</td>
</tr>
<tr>
<td>L-Lys-D-Ala-D-Ala and L-Lys-D-Ala</td>
<td>0.962*</td>
</tr>
<tr>
<td>LTA and Gly5</td>
<td>0.955*</td>
</tr>
<tr>
<td>β-RTA and L-Lys-D-Ala</td>
<td>0.946*</td>
</tr>
<tr>
<td>β-RTA and L-Lys-D-Ala-D-Ala</td>
<td>0.958*</td>
</tr>
<tr>
<td>β-RTA and Gly5</td>
<td>0.965*</td>
</tr>
</tbody>
</table>

* P > 0.05.  
* P < 0.01.  
* P < 0.001.  
* P < 0.05.
fractions of positives. Three relevant correlation plots of antibody values are presented in Fig. 2. PG was the best antigen used alone or in combination with its L-Lys-D-Ala-D-Ala epitope: the fraction of positives increased from 42 to 55%. Slightly lower percentages were found for β-RTA or LTA in combination with PG. However, such antigen combinations resulted in high percentages of positives for the blood donor control group as well. Of 31 adult patients, 12 (8 with osteomyelitis, 3 with tissue infections, and 1 with septicemia) had levels of antibodies to all antigens in the normal range. When three antigens, e.g., PG, L-Lys-D-Ala-D-Ala, and β-RTA, were combined, the maximum fraction of positives (61%) was obtained, but 37% of the blood donors were positive as well.

Predictive values. The clinical usefulness of the antigens alone or in combinations was estimated by calculating PV-P and PV-N. The results of selected tests are presented in Table 3. When one antigen, the staphylococcus-specific epitope Gly, was used, the highest PV-P (67%) and PV-N (74%) were obtained. In all circumstances, however, predictive values were low for all combinations of antigens. The highest PV-P was 58% and the highest PV-N was 77% when the criterion was a seropositive result in at least one of two tests, and the respective values were 44 and 78% when the criterion was a seropositive result in at least one of three tests. Some patients were seropositive in at least three tests. Although 100% PV-P could be obtained for one of the selected antigen combinations, the corresponding fraction of seropositive patients was low (19%).

| TABLE 3. Predictive values of antibodies against selected combinations of antigens |
|---------------------------------|-----------------|-----------------|
| Positive or negative score in:  | Antibodies to:   | % Positive      | % Negative     |
| One test                        | Gly,            | 67              | 74              |
| At least one test               | L-Lys-D-Ala-D-Ala and/or L-Lys-D-Ala | 58              | 77              |
| test                            | PG and/or β-RTA and/or L-Lys-D-Ala-D-Ala | 44              | 78              |
| All tests                       | L-Lys-D-Ala-D-Ala and L-Lys-D-Ala | 64              | 73              |
|                                | PG and β-RTA and L-Lys-D-Ala-D-Ala | 100             | 72              |
The purpose of this study was to investigate the prevalence of antibodies to the well-characterized staphylococcal cell wall antigens PG, β-RTA, and LTA and to the PG peptide epitopes in sera from blood donors and patients with staphylococcal infections. The patient group was heterogeneous with regard to both age and type of staphylococcal infections. This heterogeneity illustrates one of the problems in staphylococcal serology, as the antibody response varies with both the age and the type of infection. Another problem has been to identify test antigens which are specific for staphylococci. In this respect it would be of particular interest to study the presence of antibodies to the staphylococcus-specific epitope Gly$_c$ located in PG.

The cell wall antigens used in this study have previously been characterized in our laboratory (1, 12, 31). The synthetic peptides corresponding to the PG epitopes have all previously been shown to bind anti-PG antibodies (13, 31).

Our findings of a wide range of levels of normally occurring antibodies to PG, LTA, and β-RTA in all blood donor sera (Table 1) are in agreement with earlier findings (16, 29, 32). Others have found that such sera also contain various amounts of antibodies to the C-terminal L-Lys-D-Ala-d-Ala (10). This was also the case in this study for both patient and blood donor sera with regard to antibodies to all three PG epitopes. The levels of antibodies to the staphylococcus-specific epitope Gly$_c$ were lower than those of antibodies to both L-Lys-D-Ala-d-Ala and L-Lys-d-Ala (Fig. 1A), confirming our previous findings (31).

By our criteria less than 50% of 31 adult patients were seropositive for any of the test antigens, probably because of the low number of septicemia (n = 3) and endocarditis (n = 2) cases in our study group. In such cases of staphylococcal infections the highest levels of antibodies have been found for PG (4), β-RTA (33), and LTA (32).

The cut-off level between positive and negative antibody values in child sera could not be determined, owing to difficulty in obtaining age-matched controls. Earlier studies, however, showed that normal levels of antibodies to both PG (29) and β-RTA (16) are lowest for children under 5 years old and reach a maximum at about 20 years of age. In this study we also observed that children had lower and less-scattered antibody values than did adults.

Staphylococcus-specific IgM antibodies are generally not found or are present only at low concentrations in human sera (11, 29, 33). Consequently, we investigated the IgG response in an ELISA. The low antibody reactivity with the staphylococcal antigens found in many of the patient sera could not be attributed to low production of IgG in general, since only one of the adult patients and two of the children had below-normal levels of IgG.

Among the 26 serum specimens from children under the age of 14, 7 were from patients that did not have staphylococcal infections. None of our test antigens allowed us to discriminate between the staphylococcal and nonstaphylococcal infection groups (Fig. 1B). Except for Gly$_c$, the PG epitopes are common for staphylococcal and streptococcal PGs. Antibodies to the cell wall antigens PG (31), β-RTA (19), and LTA (32) are known to cross-react with various species because of common epitopes. The staphylococcal antibodies in sera from patients with nonstaphylococcal infections could be cross-reactive antibodies or could be the result of previous staphylococcal infections.

Two or more serum samples from each patient were analyzed, but in most cases the antibody levels remained stable throughout the observation period. This result contrasts with the findings of others demonstrating significant seroconversions in the levels of antibodies to PG (4, 27, 28) and β-RTA (27, 28) in sera from patients with serious staphylococcal infections. The differences between our data and the results of others could be the result of different times of serum sampling and the fact that the patient group in our study was heterogeneous. One may also speculate whether so-called “acute-phase” and “convalescent-phase” sera are relevant terms in serology involving a bacterial species so frequently belonging to the resident flora.

Patients with osteomyelitis do not usually have high levels of antibodies to staphylococcal cell wall antigens. A recent study (14) showed that such patients more frequently had antibodies to β-RTA than to PG. This was also the case in our study, although the fractions of positives for both antigens were low, 38 and 19%, respectively.

The positive correlations (Table 2) found between antibodies to PG and LTA and between antibodies to PG and β-RTA are in accordance with earlier findings (29, 32). These three cell wall antigens are all immunogenic and are present in considerable amounts on the bacterial surface. The observed covariation in antibody response was therefore as expected. The positive correlation observed between antibodies to LTA and β-RTA and between these and antibodies to the alanine-containing PG epitopes could also partly be the result of cross-reactive antibodies. The correlation between antibodies to PG and its peptide epitopes was statistically significant (P < 0.01), although none of the correlation coefficients were higher than 0.579. The reason might be that when whole PG is used as an antigen, antibodies reactive with the carbohydrate determinants are also detected (34).

The findings of significant differences between means of levels of antibodies to PG and its peptide epitopes in sera from adult patients and blood donors indicated that these antigens could be used for serodiagnostic purposes. However, the coefficients of variation (Table 1) ranged from 37 to 55%, indicating that the two main groups of sera could not be easily separated. The fraction of seropositive patients, which was low for each antigen used alone (<42%), increased when two (55%) or three (61%) antigens were combined. A similar increase was observed for the blood donor sera in multiple-antigen assays. Consequently, the predictive values for these staphylococcal antibodies were too low to be of diagnostic value. Disappointingly, even the staphylococcus-specific epitope Gly$_c$ did not stand out as a convincing diagnostic test antigen, as the PV-F in the single-antigen assay was 67% when 32% of the adult patients and 6% of the blood donors were seropositive. Our results, however, do not exclude the possibility that there are other staphylococcal antigens or epitopes which could perform satisfactorily for serodiagnostic purposes. Another solution might be to detect antigens instead of antibodies in biological material by use of staphylococcus-specific monoclonal antibodies.

**LITERATURE CITED**

ANTIBODIES TO STAPHYLOCOCCAL ANTIGENS


