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Antibodies to Various Bacterial Cell Wall Peptidoglycans in Human and Rabbit Sera

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Sera from patients with verified systemic staphylococcal infection contained antibodies reactive with peptidoglycan (PG) from *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Micrococcus lysodeikticus*, *Bacillus subtilis*, and *Escherichia coli*. The presence of anti-PG cross-reactive antibodies was verified in patient sera by inhibition studies with the various bacterial PGs. Antibodies to nonstaphylococcal PGs were also elevated in sera from rabbits immunized with *S. aureus* PG. Antibodies to *S. aureus* PG were removed with the synthetic peptide analogs of *S. aureus* PG, the L-Lys-D-Ala-D-Ala, L-Lys-D-Ala, and $(Gly)_5$ determinants, as well as with an *S. aureus* PG peptide fragment containing the determinants D-Ala-D-Ala and L-Lys-D-Ala. Isolated antibodies to the PG peptides, both synthetic and native, were reactive with *S. aureus* and *S. epidermidis* PGs. The antibodies to the D-Ala-D-Ala and the L-Lys-D-Ala determinants were also reactive with *S. pyogenes* PG, but not with PGs from *M. lysodeikticus*, *B. subtilis*, and *E. coli*.

Significantly elevated levels of antibody to *Staphylococcus aureus* peptidoglycan (PG) have been found in more than 70% of patients suffering from serious *S. aureus* infection (3, 4, 28, 31). However, some patients with nonstaphylococcal infections also had elevated levels of antibodies to *S. aureus* PG (4, 31). This raises the question of the specificity of the test when PG is used as the antigen.

PG is the major cell wall component in gram-positive bacteria, but constitutes less than 10% of the cell wall in gram-negative organisms. It consists of a sugar backbone covalently bound to peptide units. A detailed description of the primary structure of bacterial PGs and their variations was published by Schleifer and Kandler in 1972 (22).

Anti-PG antibodies in rabbit immune sera cross-reacting with various PGs from both gram-positive and gram-negative bacteria have been described (10–12, 17, 21, 23), showing that many bacterial species possess PGs with immunochemically similar antigenic determinants. The chemical structure of antigenic determinants of some PGs is known. *N*-Acetylglucosamine is the immunodominant sugar (6, 12, 21), but antibodies to *N*-acetylmuramic acid have also been found (33). In the peptide unit, antibodies are reactive against the pentapeptide determinant D-Ala-D-Ala and the tetrapeptide determinant L-Lys-D-Ala (8, 13, 23). *meso*-Diaminopimelic acid is located in position 3 in the PG peptide from gram-negative bacteria, and *meso*-diaminopinelic acid-D-Ala has been shown to be immunodominant in this peptide (17). In addition antibodies have been found in rabbit immune sera to the interpeptide glycine bridge in PG from *S. aureus* (8, 19, 25). Anti-PG cross-reactive antibodies in rabbit immune sera have been found to be reactive with the sugar moiety of bacterial PG (12, 23). Furthermore, anti-PG cross-reactive antibodies have been shown to be specific for tetrapeptides and pentapeptides of some bacterial PGs (8, 10, 12, 23).

Recently antibodies to *S. aureus* PG have been detected in all human sera analyzed by enzyme-linked immunosorbent assays (ELISAs) and radioimmunoassays. Verbrugh et al. (28) and Christensson et al. (3, 4) found that some patients with gram-positive infections but none with gram-negative, bacterial infections had elevated levels of anti-*S. aureus* PG antibodies. We have found that patients with gram-negative bacterial infections also have elevated levels of anti-*S. aureus* PG antibodies (31).

In this paper we present the data of analyses of cross-reactive antibodies in human and rabbit sera against various bacterial PGs.

MATERIALS AND METHODS

Preparation of PGs. PGs from *S. aureus* Cowan 1 and *Staphylococcus epidermidis* 1254 were prepared as described previously (18). PGs from *Streptococcus pyogenes* and *Bacillus subtilis* (both locally isolated), *Micrococcus lysodeikticus* NCTC 2655, and *Escherichia coli* ATCC 25922 were prepared as described previously (30). The bacteria were digested with 1% (wt/wt) trypsin and treated with 2% sodium dodecyl sulfate at 100°C for 4 min. After 12 h at room temperature, the insoluble PG residues were isolated by centrifugation. The pellets containing PG were washed twice in 2% sodium dodecyl sulfate solution and once in distilled water. The preparations were dialyzed against distilled water and freeze-dried. The PGs were hydrolyzed in HCl and analyzed by gas-liquid chromatography for amino acids (20) and carbohydrates (1) as described previously. Sugar alcohols were detected by paper chromatography (15), and phosphate was detected as described previously (2).

Preparation of PG fragments. PG (25 mg) in 0.01 M phosphate buffer (pH 7.5) containing 0.05 M NaCl was digested with 10 U of lysostaphin (specific activity, 289 U/mg; Sigma Chemical Co.). The enzymatic reaction was carried out overnight at 37°C. The digest was then centrifuged at 15,000 × *g* for 30 min. The supernatant containing the fragments was fractionated on a Sephadex G-25 superfine column (2.5 by 45 cm). The eluent was 0.05 M ammonium acetate with 0.02% NaN_3. Fractions containing PG fragments were freeze-dried before alkaline catalytic

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TABLE 1. Molar ratios of some amino acids in PGs from six species of bacteria[a]

PG source	Molar ratios of amino acids			
	Ala	Gly	Glu	Lys
S. aureus	1.8	2.7	1.1	1.0
S. epidermidis	1.3	2.5	0.8	1.0
S. pyogenes	1.1		0.5	1.0
M. lysodeikticus	3.1	0.9	1.3	1.0
B. subtilis	1.4		1.0	
E. coli	1.1		1.0	

[a] Ser was found in PG from *S. epidermidis*. Traces of Asp, Leu, and His were found in all PGs, and traces of Ser, Gly, and Lys were found in PGs from *E. coli* and *B. subtilis*.

hyrolysis was performed (27). The degraded PG was applied to a Bio-Gel P-2, 200/400-mesh column (0.5 by 115 cm; Bio-Rad Laboratories) and eluted with distilled water. The columns were calibrated with peptides of known size ranging from 174 to 5,600 daltons. Samples from fractions were hydrolyzed and analyzed for amino acids by gas-liquid chromatography. Hexosamines were determined by a modified Morgan Elson method (32). The disappearance of muramic acid after alkaline treatment of the PG was assessed by paper chromatography of amino sugars (15).

Synthetic peptides. Commercially obtained synthetic pentaglycine $(Gly)_5$ (Sigma), diacetyl-L-Lys-D-Ala-D-Ala, and diacetyl-L-Lys-D-Ala were used (Bioproducts, Belgium).

Sera. Sera from three patients with *S. aureus* septic arthritis, chronic osteomyelitis, and septicemia were examined. The diagnoses had been verified by positive blood cultures. Sera from three blood donors were also examined. Antisera to *S. aureus* PG were produced in locally bred Landrace rabbits by injecting PG and Freund complete adjuvant (Difco Laboratories) intramuscularly, followed by four injections of PG in Freund incomplete adjuvant given subcutaneously. Each dose contained 0.25 to 1.0 mg of purified PG and was given every second week. Preimmune and immune sera were collected and were immediately stored in working samples at −70°C.

ELISA. Antibodies to bacterial PG were detected by a standard ELISA procedure. Polystyrene enzyme immunoassay plates (96 wells; Costar) were coated with ultrasonicated PG (15 min at 20 kHz), diluted in 0.01 M phosphate-buffered saline (PBS; pH 7.2) to a concentration of 5 μg/ml, and left overnight with 150 μl per well. Sonication was performed to ensure a homogeneous coating suspension of the insoluble PG, thereby increasing the reproducibility of the test. After coating, the wells were washed three times with PBS containing 0.05% Tween 20 (Norsk Medisinaldepot, Norway). Serum (100 μl) diluted in PBS-Tween 20 was added to the wells and incubated at 37°C for 2 h. After three washes with PBS-Tween 20, 50 μl of diluted horseradish peroxidase-conjugated rabbit immunoglobulins to human immunoglobulin G (IgG) (γ chain) antiserum (Dako Immunglobulins A/S, Denmark) was added, and the mixture was incubated for 1 h at 37°C. Unbound conjugate was removed by washing three times with PBS-Tween 20. The substrate (100 μl) was then added (40 mg of *o*-phenylenediamine dihydrochloride [Sigma] and 40 μl of H_2O_2 in 100 ml of sodium phosphate-citrate buffer [pH 5.0]). The enzymatic reaction lasted for 10 min at room temperature and was stopped by adding 50 μl of 2.5 N H_2SO_4. The optical density (OD) was read at 492 nm on a Titertek Multiscan Spectrophotometer (Flow Laboratories, United Kingdom).

Inhibition studies. PGs used in inhibition studies were ultrasonicated for 30 s at 20 kHz. The experiments were performed with serum dilutions corresponding to the linear component of the serum titration curves to the target antigens. Antibody solutions were incubated with PG for 4 h at room temperature and overnight at 4°C with slow agitation. The solutions were tested in ELISA for remaining antibody activity.

Immunosorbents. Synthetic peptides and a PG peptide fragment were each coupled to cyanogen bromide-activated Sepharose 4B by the free amino group of the peptides. The couplings were performed as described by the manufacturer (Pharmacia Fine Chemicals AB, Sweden). The Sepharose

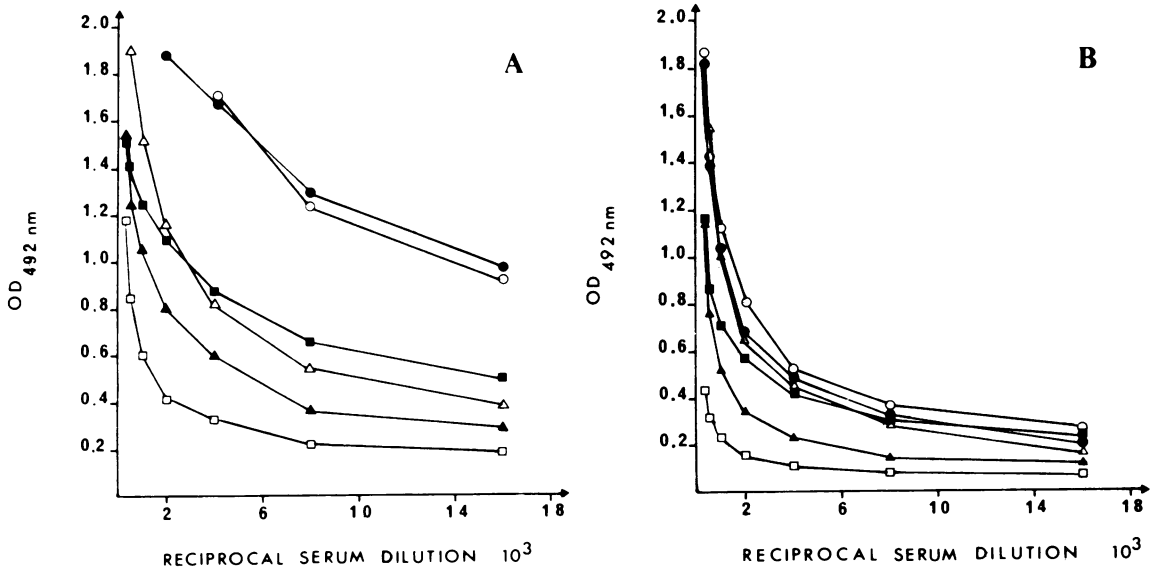

FIG. 1. Levels of antibody to PG from *S. aureus* (○), *S. epidermidis* (●), *S. pyogenes* (△), *M. lysodeikticus* (▲), *B. subtilis* (□), and *E. coli* (■) determined by ELISA in (A) serum from a patient with *S. aureus* septicemia and (B) serum from a blood donor.

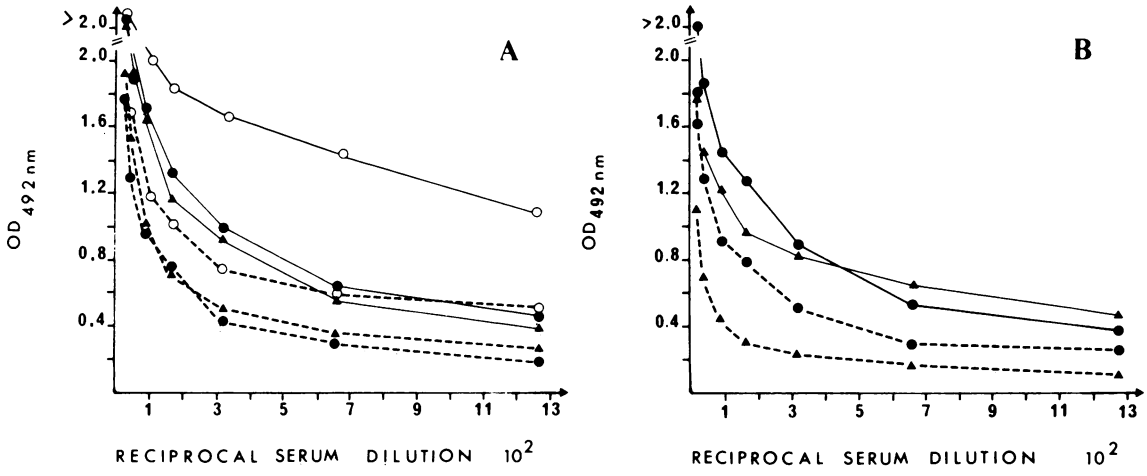

FIG. 2. Antibody levels in rabbit preimmune serum (- - -) and rabbit anti-*S. aureus* PG immune serum (——) determined by ELISA. Antigens: (A) PG from *M. lysodeikticus* (●), *B. subtilis* (▲), and *S. aureus* (○); (B) PG from *S. pyogenes* (▲) and *E. coli* (●).

peptide conjugates were packed into columns. The patient sera with high titers against *S. aureus* PG were absorbed. After extensive washing with PBS, absorbed specific antibodies were eluted with 3 M NaSCN (pH 7.0). The eluates were immediately dialyzed against PBS before the antibody activities were determined by ELISA.

RESULTS

Purity of PG preparations. The molar ratios of the amino acids in the PGs are presented in Table 1. The results are in accordance with earlier analyses (7, 22); only traces of contaminating amino acids were present. Ribitol and glycerol were not detected by paper chromatography analysis, and the phosphate content was less than 1% (wt/wt) in all PGs, showing that no significant contamination by teichoic acid or lipoteichoic acid existed.

Peptide fragment from *S. aureus* PG. Amino acid analysis of PG fragments isolated from gel filtrates of lysostaphin- and alkaline-treated PG showed fractions containing only the amino acids Ala, Gly, Glu, and Lys. This product is called the PG peptide fragment in the present article.

Antibodies to PG. Figure 1 shows the levels of antibodies to PG from *S. aureus*, *S. epidermidis*, *S. pyogens*, *M. lysodeikticus*, *B. subtilis*, and *E. coli* in the serum from one patient with *S. aureus* septicemia (Fig. 1A) and in the serum from one blood donor (Fig. 1B). Figure 1 is representative for the patient and the blood donor sera tested. The serum from the patient with staphylococcal septic arthritis, however, had antibody levels to *S. pyogenes* that were higher than expected. Figure 2 illustrates the antibody levels in rabbit preimmune sera and *S. aureus* PG immune sera to *S. aureus* and to nonstaphylococcal PGs. After immunization with *S. aureus* PG, antibody reactivities to homologous and heterologous PGs were elevated.

Specificity of the PG ELISA. The results from one inhibition study are presented in Fig. 3. In all six PG ELISAs, the antibody activities could be inhibited by using a PG inhibitor that was homologous to the target antigen.

Demonstration of cross-reactive antibodies. The results from a study of cross-reactive antibodies in a patient serum are presented in Table 2. PG inhibitors heterologous and homologous to the target antigen were used, and the remaining antibody activities were tested by ELISA. The inhibitions were performed at dilutions as described above. The concentration of inhibitor was 50 μg/700 μl and was chosen from the results of previous inhibition experiments. The analysis showed that the PG inhibitor homologous to the target antigen always gave the strongest inhibition. Heterologous PGs also always reduced the activity of antibody to any of the target antigens. The PG from *S. aureus* and *S. epidermidis* gave similar inhibition, indicating shared antigenic determinants. The chemical analyses of the two PGs showed very similar chemical compositions. The results shown in Table 2 reveal that the staphylococcal PGs, as well as the other nonstreptococcal PGs, only partly inhibited the antibody reaction to *S. pyogenes* PG. Thus antibodies spe-

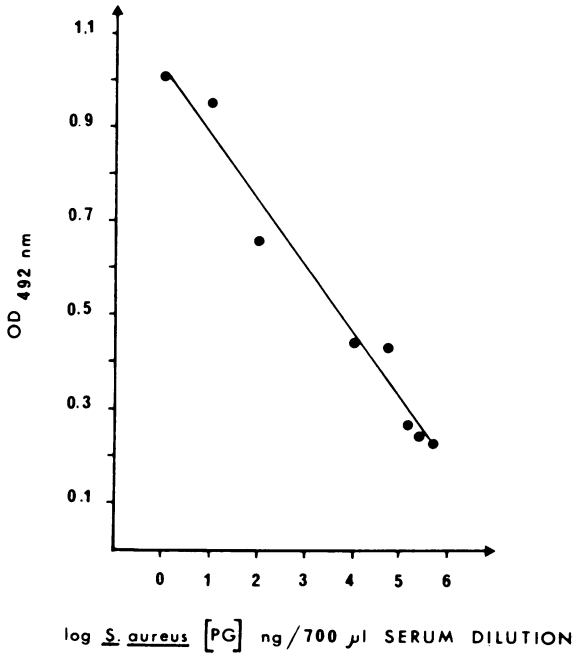

FIG. 3. Competitive inhibition studies of *S. aureus* PG reactive antibodies by homologous PG measured by ELISA in serum from a patient suffering from *S. aureus* septic arthritis.

TABLE 2. Inhibition of antibodies to various bacterial PGs[a]

Target antigen	ELISA values (OD at 492 nm) (10^2) with the following PG antigen used as inhibitor:						
	No inhibitor	*S. aureus*	*S. epidermidis*	*S. pyogenes*	*M. lysodeikticus*	*B. subtilis*	*E. coli*
S. aureus	115[b]	27	38	79	76	73	73
S. epidermidis	107[b]	48	32	80	67	74	68
S. pyogenes	119[b]	99	106	17	106	101	107
M. lysodeikticus	145[c]	101	107	118	39	110	89
B. subtilis	82[d]	79	75	87	69	28	67
E. coli	143[c]	99	92	117	94	90	37

[a] Serum from a patient with *S. aureus* septic arthritis was examined by ELISA before and after inhibition with homologous and heterologous PGs. The concentration of inhibitors was 50 μg/700 μl of diluted serum.
[b] Serum was diluted 1:3,000.
[c] Serum was diluted 1:500.
[d] Serum was diluted 1:250.

cific for *S. pyogenes* PG, either in considerable amounts or of high affinity, were probably present in the patient sera.

Study of the PG-peptide specific antibodies. Sera were absorbed on five different immunosorbents with covalently bound synthetic peptides, the L-Lys-D-Ala-D-Ala, L-Lys-D-Ala, $(Gly)_5$, and an *S. aureus* PG peptide fragment. All peptides were coupled through the NH_2 group with the COOH terminal left free. The antibody activity was tested before and after absorption. The results of the absorption of serum from one patient are presented in Table 3. The antibody activity was most reduced after absorption with the pentapeptide determinant D-Ala-D-Ala. The PG peptide fragment contained the determinants in the *S. aureus* PG peptide. Absorption with this fragment and the tetrapeptide determinant L-Lys-D-Ala reduced the antibody activity to the same level. Absorption with $(Gly)_5$ with the COOH terminal left free showed that there were antibodies present in the patient sera reactive with this peptide, which is identical to the interpeptide bridge in *S. aureus* PG. Similar experiments were performed with anti-*S. aureus* PG immune sera. In these sera most antibodies were absorbed with the PG peptide fragment isolated from the PG used for immunization. There were no differences between the tetrapeptide and pentapeptide determinants. In the patient sera, absorption with $(Gly)_5$, with the COOH terminal left free, gave a reduction in the anti-*S. aureus* PG activity.

The PG specificities of the anti-peptide antibodies in the serum from the patient with *S. aureus* septic arthritis are presented in Table 4. The antibodies to the *S. aureus* peptide, represented by the synthetic L-Lys-D-Ala-D-Ala and L-Lys-D-Ala and the PG peptide fragments, were all reactive with the staphylococcal and streptococcal PGs, but not with PG from *M. lysodeikticus*, *B. subtilis*, and *E. coli*. Antibodies to the staphylococcal interpeptide bridge $(Gly)_5$, with the COOH terminal left free, were only reactive with the staphylococcal PGs. The interpeptide bridge in *S. epidermidis* differs from that in *S. aureus* in containing serine in addition to glycine (22).

TABLE 3. Levels of antibody to *S. aureus* PG in a serum sample[a]

Immunosorbent	ELISA values (OD at 492 nm) (10^2) at the following serum dilutions:				
	1:250	1:500	1:1,000	1:2,000	1:4,000
None	200	195	165	117	82
L-Lys-D-Ala-D-Ala	140	99	165	44	31
L-Lys-D-Ala	186	164	67	87	59
S. aureus PG peptide fragment	195	172	125	88	64
$(Gly)_5$	200	169	125	88	60

[a] Serum was collected from a patient with *S. aureus* septic arthritis and examined before and after absorption with synthetic peptide analogs of the *S. aureus* PG peptide and with an *S. aureus* PG peptide fragment.

DISCUSSION

All the sera tested, for humans and rabbits, contained antibodies reactive with PG from *S. aureus*, *S. epidermidis*, *S. pyogenes*, *M. lysodeikticus*, *B. subtilis*, and *E. coli*. The raised activities of antibodies to all PGs in sera of patients, as compared with blood donor sera, were apparently due to active *S. aureus* infections. Production of antibodies to PGs other than that used for immunization of the rabbits must be due to shared antigenic determinants of the bacterial PGs tested.

The results shown in Table 2 indicate the presence of cross-reactive antibodies in serum samples from patients. The inhibition studies also showed differences between the PG preparations. Addition of the same amount of the various PG inhibitors to the sera resulted in different degrees of inhibition of activity of the antibody to the target PG used in the ELISA experiments. This indicates that varied amounts of common determinants as well as different antigenic determinants might be present.

The chemical analyses of the PG preparations showed that the amino acid and amino sugar contents corresponded well with the proposed structures. Although traces of contaminating protein were present, the preparations were relatively pure. The inhibition results could therefore not be explained by unspecific anti-PG activity.

Table 3 shows the results obtained by absorption of a patient serum with the synthetic peptides and *S. aureus* PG fragment. The results indicate that the synthetic and native peptides had the same capacity to absorb antibodies. Table 4 shows that all of the four anti-peptide antibodies isolated were reactive against the staphylococcal PGs. These antibodies, with the exception of the anti-$(Gly)_5$ antibodies, were reactive also with PG from *S. pyogenes*. As shown earlier (2) the anti-$(Gly)_5$ antibodies were only reactive with the staphylococcal PGs. Cross-reactive antibodies to streptococcal and staphylococcal PG in various rabbit immune sera have been described (12). Because of the lack of D-Ala-carboxypeptidase in staphylococci and streptococci, both tetrapeptides and pentapeptides exist in these bacteria (5). Antibodies to the pentapeptide were not reactive with PG from *B. subtilis*, *E. coli*, and *M. lysodeikticus*. Studies of *B. subtilis* PG have shown that the PG is completely cross-linked (5). *E. coli* and *M. lysodeikticus* PGs contain no pentapeptide (16, 24). Antibodies to the tetrapeptide deter-

TABLE 4. Reactivities of isolated *S. aureus* PG peptide specific antibodies determined by ELISA against various bacterial PGs[a]

Immunosorbent-isolated antibody	ELISA values (OD at 492 nm) (10^2) with target PG from:					
	S. aureus	*S. epidermidis*	*S. pyogenes*	*M. lysodeikticus*	*B. subtilis*	*E. coli*
Anti-L-Lys-D-Ala-D-Ala	63	73	32	14	4	10
Anti-L-Lys-D-Ala	73	82	30	16	4	11
Anti-*S. aureus* PG peptide fragment	63	66	40	15	10	14
Anti-$(Gly)_5$	41	50	3	10	5	11

[a] Antibodies were isolated from 0.6 ml of serum and tested in a 1:20 dilution.

minant L-Lys-D-Ala were not reactive with PGs from *B. subtilis* and *E. coli*, since these bacteria contain *meso*-diaminopimelic acid in position 3 of their PG peptide. PG from *M. lysodeikticus* contains the same tetrapeptide as the staphylococcal and streptococcal PGs, with the exception of a Gly substitute on Glu in position 2 in the peptide unit. The PG in this organism is exceptional in that it is cross-linked by a peptide identical to its peptide unit (24). The antigenic determinants in the *M. lysodeikticus* PG peptide remain to be determined. In rabbit antisera, antibodies are mainly reactive with the sugar moiety of PG from this organism (33). From our studies it seems that the cross-reactive antibodies in sera from patients with *S. aureus* infections are reactive against the sugar moiety of PG from *M. lysodeikticus*, *B. subtilis*, and *E. coli*, since none of the peptides homologous to the antigenic determinants in *S. aureus* PG peptide would bind antibodies reactive with *M. lysodeikticus*, *B. subtilis*, and *E. coli* PG.

In conclusion, our findings of PG cross-reactive antibodies in human sera are consistent with earlier reports of cross-reactive anti-PG antibodies in rabbit immune sera. Such cross-reactive antibodies may obviously cause problems in the determination of species-specific anti-PG antibodies in human sera. We have shown earlier that antibodies cross-react with lipoteichoic acid from *S. aureus* and *S. pyogenes* (31). In addition teichoic acid from various bacterial species may also contain common determinants (14).

Bacterial PG is known to have many biological properties (9). Opsonic activity of anti-PG antibodies has been reported (29), and the cross-reactive and specific antibodies to PG may have such activity. Since PG is exposed on the surface of the bacterial cell (26), both PG-cross-reactive and specific antibodies may have a function in the defense against bacterial infections. Further analyses of the biological properties of these antibodies should be performed to give an increased understanding of their activity in vivo.

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