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Analysis by Gel Electrophoresis, Western Blot, and Peptide Mapping of Protein A Heterogeneity in *Staphylococcus aureus* Strains

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To evaluate potential differences in protein A among *Staphylococcus aureus* strains, lysostaphin-solubilized cell wall proteins from 12 serologically distinct strains were analyzed by 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Seven presumptive protein A variants identified in the 45- to 57-kilodalton range were then studied for qualitative binding affinities to nonimmune mouse and rabbit immunoglobulin G (IgG) by enzyme-linked immunoelectrotransfer blot. Essentially, all presumptive protein A variants demonstrated binding to both nonimmune rabbit and mouse IgG and had differential binding to mouse monoclonal IgG1 at pH 8.2 than at 5.5. Because of Fc-binding properties and molecular weight similarity to the well-characterized Cowan I protein A, these proteins appeared to represent protein A variants. Amino sugar analysis (<1%) by reverse-phase high-pressure liquid chromatography suggested that the apparent molecular weight differences in protein A were not due to associated mucopeptides. Further differences in protein A variants were studied by peptide mapping. Each of the seven protein A variants, distinguishable on SDS-PAGE, also produced distinct peptide cleavage patterns. In addition, two protein A variants indistinguishable on SDS-PAGE could be further subdivided by peptide mapping. These results suggest that SDS-PAGE analysis of protein A, particularly in conjunction with peptide mapping, may be useful in distinguishing distinct strains of *S. aureus*. Different protein A variants may also have unique functional or immunologic capabilities.

Many bacterial species have receptors on their cell surfaces for different mammalian plasma proteins. The best-characterized of these receptors is protein A (SpA) from *Staphylococcus aureus*. SpA has the ability to bind to the Fc portion of a variety of mammalian immunoglobulins and, because of this property, is useful in a variety of immunological techniques (11). SpA is also known to activate serum complement (12, 14, 27).

Almost all studies on the functional characteristics of SpA have been performed with a single *S. aureus* strain, Cowan I, which produces this protein in abundance. Using radiolabeled immunoglobulin, Kronvall et al. estimated the number of SpA molecules on *S. aureus* Cowan I to be 80,000 immunoglobulin G (IgG)-binding sites per organism (19). Although it has been suggested that there are differences in size and amino acid composition of SpA among *S. aureus* strains (30), it has been widely assumed that SpA from different strains are similar. Our current studies of the cell wall proteins of 12 *S. aureus* strains revealed heterogeneity of a major cell wall protein in the 45- to 57-kilodalton molecular size range on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The proximity of the molecular sizes of these proteins to that of the standard Cowan I SpA (ca. 56 kDa by SDS-PAGE [2]) suggested that these bands might represent SpA variants. In this report, we describe the size variation of SpA molecule among 12 serologically distinct *S. aureus* strains and further analyze the heterogeneity of these proteins by peptide mapping.

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MATERIALS AND METHODS

Bacterial strains. Twelve serologically distinct *S. aureus* strains (kindly provided by P. Oeding, University of Bergen, Bergen, Norway) were used in the evaluation of SpA heterogeneity. These strains were isolated from humans and cultured; they have unique surface agglutinogens, which are presumed to be proteins (15, 16).

Bacterial growth and harvest. Briefly, 15 ml of an overnight culture of *S. aureus* was inoculated into 500 ml of peptone yeast extract broth (5 g of peptone, 5 g of yeast extract, and 3 g of K_2HPO_4 in 1 liter of H_2O adjusted to pH 7.5) and incubated for 11 h at 37°C with vigorous shaking. The cells were harvested by centrifugation (2,000 × *g* for 10 min) and washed three times with Tris buffer (0.05 M; pH 7.8).

Cell wall isolation and extraction. Isolation and extraction of cell walls were carried out as previously described (A. L. Cheung, A. S. Bayer, J. Peters, E. R. Kost, and J. I. Ward, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, C175, p. 329; submitted for publication). Briefly, harvested cells were disrupted by 0.1-mm glass beads in a Braun homogenizer (B. Braun Co., Melsungen, Federal Republic of Germany) for 4.5 min. The homogenized suspension was then heated in a 75°C water bath for 10 min to inactivate cell wall autolytic activity. Differential centrifugation was then carried out, twice at low speed (2,000 × *g* for 10 min) to remove intact cells and then once at high speed (15,000 × *g* for 20 min) to recover cell walls. The cell walls were then washed four times with 5 ml of Tris buffer (0.05 M; pH 7.8 with 1 M NaCl) (25).

Cell walls were then extracted twice with 5 ml of 2% Triton X-100 at room temperature for 30 min to remove contaminating cell membrane constituents (20). The cell

walls were pelleted after each Triton X-100 extraction. Lysostaphin (Sigma Chemical Co., St. Louis, Mo.) dissolved in Tris buffer (0.05 M; pH 7.8 with 0.145 M NaCl) was then added to the cell wall suspension to a final concentration of 50 μg/ml, and the mixture was incubated at 37°C for 2 h. After removal of the insoluble material by centrifugation (45,000 × *g* for 20 min), the supernatant was dialyzed and lyophilized. The protein concentration of each sample was then adjusted to 2 mg/ml for further analyses.

Assay for proteinase. Azocoll (Sigma), a proteolytic substrate, was used to determine proteolytic activity as described by Chavira et al. (4). Samples (50 μl each) from each step of the extraction method were assayed. Dilutions of trypsin (0.1 μg/ml to 1.0 mg/ml) were used as standards.

SDS-PAGE. PAGE was performed by the procedure described by Laemmli (21). Samples in dissociating buffer (21) were boiled for 5 min before being loaded onto the gel. After electrophoresis, the gels were stained with Coomassie brilliant blue R250 (31).

Enzyme-Linked Immunoelectrotransfer Blot. Cell wall proteins from 12 serologically distinct *S. aureus* strains were separated on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose as described by Towbin et al. (29). After transfer, the nitrocellulose membrane was immersed overnight in blocking buffer (0.5% [vol/vol] Tween, 1.0 M NaCl, 0.01 M Tris [pH 8.2], 0.02% NaN_3) and then washed for 30 min in blocking buffer before primary antibody was added. Nitrocellulose membranes were probed separately as follows: (i) nonimmune mouse IgG (Calbiochem-Behring, La Jolla, Calif.), 50 μg in 50 ml of incubation buffer (0.05% Brij 35, 0.15 M NaCl, 0.01 M Tris [pH 8.2], 0.02% NaN_3 for 2 h; (ii) nonimmune rabbit IgG (Cappel Laboratories, Cochranville, Pa.), 50 μg in 50 ml of incubation buffer for 2 h; (iii) mouse monoclonal IgG_1 to α_1-antitrypsin (Cappel Laboratories) diluted 1:500 in 50 ml of incubation buffer (pH 8.2) for 3 h; and (iv) mouse monoclonal IgG1 diluted 1:500 in 50 ml of 0.01 M phosphate buffer (pH 5.5) with 0.05% Brij 35, 0.15 M NaCl, and 0.02% NaN_3 for 3 h. The blots with the mouse monoclonal antibody were designed to demonstrate nonimmune Fc binding of these proteins. After probing, the nitrocellulose membrane was washed three times for 5 min each with blocking buffer. Alkaline phosphatase conjugate of the $F(ab')_2$ fragment of sheep anti-mouse or sheep anti-rabbit IgG (Sigma) was added as the secondary antibody (dilution 1:1,000). After incubation for 1 h, the nitrocellulose membrane was again washed three times for 5 min each with blocking buffer, and the reactive bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as the alkaline phosphatase substrate as described by Blake et al. (3).

Periodic acid-Schiff base stain for glycoprotein. Cell wall proteins from 12 serologically distinct *S. aureus* strains together with ovalbumin as a positive control were resolved on 7.5% gels. After being fixed overnight in ethanol-glacial acetic acid-water (40:5:55), the gel was oxidized by periodic acid and reacted with dansyl hydrazine to form a Schiff base as described by Eckhardt et al. (7). Following reduction by sodium borohydride, the gel was treated with acetic acid-water (99:1) repeatedly until the background became colorless. After being destained, fluorescent-labeled glycoproteins were visualized with a long-range UV light (366 nm). Control gels in which the periodate oxidation step was omitted were included to reveal proteins with natural fluorescence or those that bound dansyl hydrazine noncovalently.

Amino sugar analysis. To quantitate the amount of amino sugar in our protein A variants, protein A was first purified on a IgG-Sepharose affinity column (17). About 5 μg of the purified protein was hydrolyzed wiht 4 N HCl at 100°C for 7 h in a PicoTag Work Station (Waters Associates, Inc., Milford, Mass.). The hydrolyzed sample was then derivatized with phenylisothiocyanate (1). The phenylthiocarbamyl derivatives of the amino sugar were eluted from a Novapak C_{18} (3.9 mm by 15 cm) column (Waters Associates) in a high-pressure liquid chromatography unit (Waters Associates) with a convex gradient of 10% to 51% solvent B in 10 min. The solvent system consisted of two eluants: solvent A (0.14 M sodium acetate [pH 5.3]) and solvent B (60% acetonitrile in water). The eluants were detected with a multiwavelength monitor at 254 nm. A standard of glucosamine was run simultaneously.

Peptide mapping. To further evaluate the differences in protein A, peptide mapping was performed by the method of Cleveland et al. (5). Briefly, sections of gel containing the stained SpA variant were cut, equilibrated with stacking buffer (0.125 M Tris [pH 6.8], 0.1% SDS), and set into a sample well of a second SDS-gel. The second gel consisted of a lower-resolving gel of 15% or 12.5%, while the staking gel contained 6% acrylamide with 1 mM EDTA. The gel slices were then overlaid with either 5 μg of chymotrypsin (type 1S; Sigma) or 0.5 μg of *S. aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.) in sample buffer (0.125 M Tris [pH 6.8], 0.1% SDS, 10% glycerol, 0.002% bromophenol blue). Electrophoresis was performed at constant voltage (50 V) through the stacking gel until the protease and polypeptide were compressed into a band in the stacking gel. At that time, the power was turned off for 30 min to allow for further enzymatic digestion. Electrophoresis was then continued at constant voltage (150 V) until the dye front reached the bottom of the gel. The gels were then stained with Coomassie blue (31).

RESULTS

Cell wall protein profiles by SDS-PAGE. Cell-wall proteins of 12 serologically distinct *S. aureus* strains were resolved by SDS-PAGE (7.5% gel). The major protein bands were in the molecular size range of 45 to 75 kDa (Fig. 1). Although electrophoretically distinct from the Cowan I SpA control (lane 15), the quantitative predominance of this band in each of the protein profiles and the proximity of its molecular size to that of Cowan I SpA suggested that these bands might represent SpA variants.

Identification of protein A by enzyme-linked immunoelectrotransfer (Western) blots. To more precisely identify SpA among the 12 *S. aureus* strains examined, cell wall proteins from these strains were resolved on 7.5% gels and transferred to nitrocellulose membranes. All of the presumed SpA variants (except for *S. aureus* 5687 [lane 6], which displayed weak binding), including the lower-molecular-size polypeptides at the migration front of each lane, exhibited binding to nonimmune rabbit IgG (Fig. 2). Similar results were also found with nonimmune mouse IgG (data not shown). When the blot was probed with mouse monoclonal IgG1 at pH 8.2, all of the suspected SpA variants except one (strain 5687) demonstrated binding (data not shown). In contrast, incubation of the blot with the same monoclonal antibody at pH 5.5 revealed little or no binding. Furthermore, a blot with alkaline phosphatase conjugates of sheep anti-mouse IgG revealed binding to almost all of the suspected SpA variants, while the blot with alkaline phosphatase conjugates of sheep anti-mouse $F(ab')_2$ did not bind. These data suggested that the protein of interest had Fc-

binding properties similar to that of Cowan I SpA and thus appeared to represent SpA size variants of different molecular size. In addition, polypeptides found at the gel front had nonspecific IgG-binding properties.

By SDS-PAGE with Coomassie blue and silver stain (data on silver stain not shown), there appeared to be at least six distinguishable SpA size variants among the 12 strains studied (Fig. 1). To facilitate the interpretation of data, we arbitrarily assigned SpA variants of similar size by SDS-PAGE to groups. With mixing experiment, we found that group I strains were different from group III strains and, further, that SpA from strain 2253 (Fig. 1, lane 3) appeared to be distinct from other members within the same group (group I). To evaluated the possibility that the apparent molecular size differences in SpA were due to various amounts of associated mucopeptide attached to a homogeneous SpA molecule, we stained the gels for glycoproteins as described. No fluorescence-labeled glycoproteins were detected in the putative SpA variants, although the ovalbumin control was positive for fluorescence. Further quantitation of amino sugars was performed with two of our purified SpA variants (strains 17A and 263) by reverse-phase high-pressure liquid chromatography. This method allowed for detection of amino sugars down to the 10-pmol level. Our results indicate that less than 1% amino sugar was present.

Proteolytic activity. To evaluate the possibility that these SpA size variants might have resulted from proteolytic digestion of a single SpA molecule, we monitored proteolytic activity by using Azocoll at each step of the preparation of the cell wall proteins. A minimal amount of proteolytic activity was detected by the Azocoll assay (A_{520} = 0.025) and was detected only during the initial cell disruption. This degree of protease activity is equivalent to that of 0.1 μg of trypsin per ml.

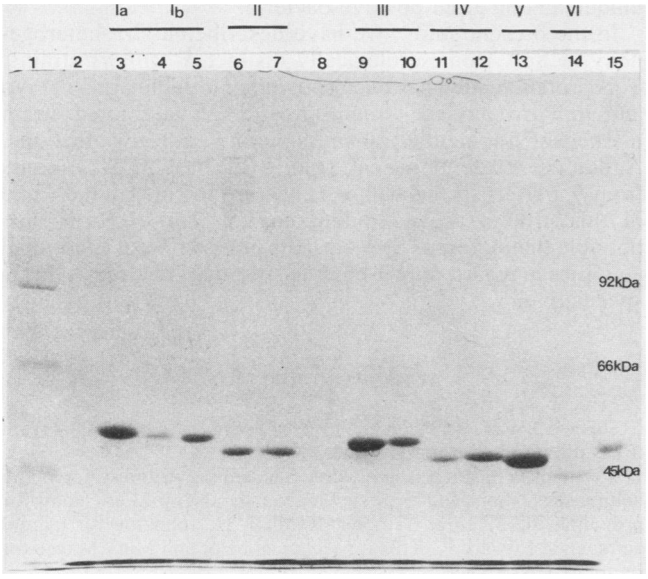

FIG. 1. SDS-gel (7.5%) of cell wall extracts from 12 serologically distinct *S. aureus* strains. Roman numerals denote electrophoretic variants of SpA of similar size. Lanes: 1, molecular size standards (phosphorylase *b*, bovine serum albumin, and ovalbumin); 2, lysostaphin control; 3, *S. aureus* 2253; 4, *S. aureus* Cowan III; 5, *S. aureus* 2095; 6, *S. aureus* 21; 7, *S. aureus* 17A; 8, *S. aureus* 5687; 9, *S. aureus* 1503; 10, *S. aureus* 3647; 11, *S. aureus* 670; 12, *S. aureus* 3189; 13, *S. aureus* 6376; 14, *S. aureus* 263; 15, standard Cowan I protein A. Electrophoresis was performed at constant voltage (50 V in the stacking gel and 150 V in the resolving gel). The gel was stained with Coomassie brilliant blue R250. Cell wall proteins of *S. aureus* 5687 (lane 8), poorly stained in this gel, were clearly visible on a silver stain (gel not shown).

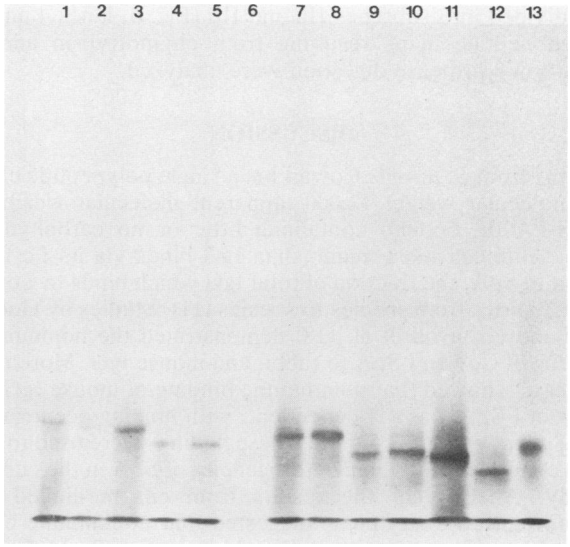

FIG. 2. Western blot of cell wall lysostaphin extracts of 12 *S. aureus* strains probed with nonimmune rabbit IgG. Lanes: 1, *S. aureus* 2253; 2, *S. aureus* Cowan III; 3, *S. aureus* 2095; 4, *S. aureus* 21; 5, *S. aureus* 17A; 6, *S. aureus* 5687; 7, *S. aureus* 1503; 8, *S. aureus* 3647; 9, *S. aureus* 670; 10, *S. aureus* 3189; 11, *S. aureus* 6376; 12, *S. aureus* 263; lane 13, Cowan I protein A control. Each lane was loaded with ca. 0.5 μg of protein.

Peptide mapping. To confirm size heterogeneity of SpA among 12 *S. aureus* strains, we studied the proteins by peptide mapping. SpA (approximately 20 μg in each lane) was digested with chymotrypsin or *S. aureus* V8 protease, and the cleavage fragments were analyzed by SDS-PAGE (15 or 12.5%). Each of seven SpA variants distinguishable as intact proteins on SDS-PAGE yielded distinct peptide cleavage patterns (Fig. 3; data on *S. aureus* V8 protease not shown). In addition, one SpA variant (II) cold be further

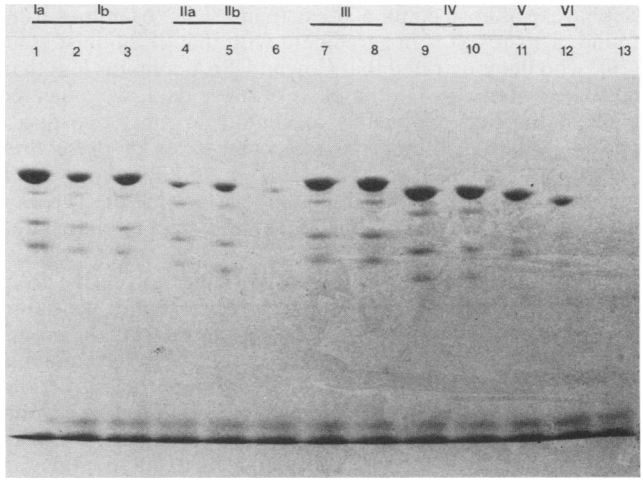

FIG. 3. Peptide mapping gel (15%) of protein A from 12 serologically distinct strains with 5 μg of chymotrypsin. The lanes are the same as in Fig. 2, except for lane 13, which contained 5 μg of chymotrypsin control.

subdivided into subtypes IIa and IIb (Fig. 3, lanes 4 and 5) when peptide maps resulting from chymotrypsin and *S. aureus* V8 protease digestion were analyzed.

DISCUSSION

SpA from *S. aureus* Cowan I is a single polypeptide chain of molecular weight 42,000 (apparent molecular weight in SDS-PAGE, 56,000) containing little or no carbohydrate (11). Although most mammalian IgG binds via its Fc fragment to SpA, the fraction of total IgG which binds to Cowan I SpA varies from species to species (11). Studies by Goding (10) and Kronvall et al. (18) demonstrated the nonimmune bindng of Cowan I SpA to rabbit and mouse IgG. Moreover, Ey et al. showed that nonimmune binding of mouse IgG1 to Cowan I SpA was pH dependent, with binding occurring at pH 8.0 but not at pH 5.5 (8). These findings were used to test and confirm the possible heterogeneity of SpA in the current study. Analysis of the results from enzyme-linked immunoelectrotransfer blots suggested that nonimmune binding of these proteins to both rabbit and mouse IgG was occurring. In addition, these proteins exhibited differential binding to monoclonal mouse IgG1 at different pH conditions as described (8). Furthermore, these proteins displayed binding to alkaline phosphatase conjugates of intact sheep IgG but not the $F(ab')_2$ fragments. These results indicate that these proteins are SpA, which vary in apparent molecular weight.

Previous studies showed that lysis of staphylococcal cells by lysostaphin was due to hydrolysis of the peptidoglycan in the cell wall and was catalyzed by a glycosidase and a peptidase. Subsequent investigation by Tipper and Strominger demonstrated that the glycosidase, in conjunction with the peptidase, liberates a mixture of disaccharides, some of which are sensitive to periodate oxidation (28). With the assumption that one protein A molecule is covalently linked to one dissacharide, there should be approximately 200 ng of disaccharide in 20 μg of SpA. This amount of carbohydrate would be detected by the periodic acid-Schiff stain described above if the sensitivity of this stain with mucopeptides was comparable to that of ovalbumin (7). We also performed hexosamine analysis on SpA variants from strains 17A and 263 (Fig. 1, lanes 7 and 14) to determine the quantities of mucopeptides after purification on an IgG-Sepharose affinity column. Less than 1% hexosamines were found. This result is in agreement with that of Sjöquist et al. (26), who demonstrated that Cowan 1 SpA contains less than 0.2% hexosamines. On the basis of these data, we expected each of the SpA molecules to contain at most two disaccharide subunits. Since the molecular sizes of these SpA variants (strains 263 and 17A) differ by 5 kilodaltons when measured by SDS-PAGE, it is unlikely that the discrepancy in their apparent molecular sizes was due to associated mucopeptides.

To confirm that these SpA variants did not derive from proteolytic digestion of the same SpA molecule, we monitored the proteolytic activity during the extraction procedure. Proteolytic assays with Azocoll detected minimal protease activity only during the first step of the extraction. However, further analysis of the enzyme-linked immunoelectrotransfer blots (Fig. 2) revealed significant binding activities from lower-molecular-weight polypeptides at the dye front of each lane. These polypeptides may represent degradation products of SpA by an enzyme or enzymes not readily detected by Azocoll. Notably, commercially available SpA (Fig. 2, lane 13) also contained these small peptides. It seems likely that proteolytic activity accounted for the presence of these polypeptides. Nevertheless, it is unlikely that differences in the apparent molecular weight observed with SpA could be explained by proteases alone.

Previously, the content of SpA in extracts of staphylococcal cells has been estimated by single radial immunodiffusion (23), hemagglutination (27), solid-phase radioimmunoassay (6, 22), or, recently, enzyme-linked immunosorbent assays (24). These methods are all based on the nonimmune binding of SpA to IgG. Since almost all SpA from different *S. aureus* strains bind to mammalian IgG, it has been assumed that SpA from different strains are the same. Our studies of the cell wall proteins of *S. aureus* provide evidence that SpA variants among serologically distinct strains of *S. aureus* can differ by as much as 12 kilodaltons in apparent molecular size. Passage experiments in vitro and in vivo suggest that SpA is stable (Cheung et al., submitted). Analysis of these SpA variants by peptide mapping has also shown distinct peptide cleavage patterns in SDS-gels for most of these proteins. Recent studies by Guss et al. (13) demonstrated unique C-terminal amino acid sequences in SpA from two strains of *S. aureus*, implying possible heterogeneity of SpA in amino acid content. Further studies by Uhlén et al. have elucidated the complete nucleotide sequence of the gene encoding protein A from strain 8325-4 (30). The size of the deduced protein A from strain 8325-4 is larger than those from Cowan I and strain A676 derived from the molecular determinations of the purified proteins. Fischetti et al. also found that M-protein molecules in group A streptococci can vary with respect to molecular size both between M types and within an M type (9). Therefore, heterogeneity in molecular size of a major cell wall protein in *S. aureus* is not unique among gram-positive bacteria.

In the present study, we have described a size heterogeneity in SpA from serologically distinct *S. aureus* strains. This protein, which has been shown to be stable upon in vivo and in vitro passage, might provide an additional strain marker for tracing the transmission of *S. aureus* infections. Additional epidemiological studies of protein A variants from *S. aureus* strains will be necessary to confirm the value of this protein as an epidemiological marker. It is also possible that different SpA variants have different functional activities in regard to IgG-binding specificity, although this is speculative.

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