Antibody Responses in Patients with Staphylococcal Septicemia against Two *Staphylococcus aureus* Fibrinogen Binding Proteins: Clumping Factor and an Extracellular Fibrinogen Binding Protein

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We analyzed the serum antibody responses against two *Staphylococcus aureus* fibrinogen binding proteins, the cell-bound clumping factor (Clf) and an extracellular fibrinogen binding protein (Efb). The material consisted of 105 consecutive serum samples from 41 patients suffering from *S. aureus* septicemia and 72 serum samples from healthy individuals. An enzyme-linked immunosorbent assay (ELISA) was developed. Healthy individuals showed variable levels of antibodies against the studied antigens, and cutoff levels (upper 95th percentile) against these antigens were determined. No correlation was seen between serum antibody levels against Clf and Efb. In acute-phase samples 27% of patients showed positive antibody levels against Clf and 10% showed positive levels against Efb, while in convalescent-phase samples 63% (26 of 41) showed a positive serology against Clf and 49% (20 of 41) showed a positive serology against Efb. Antibody levels against Efb were significantly lower in the acute-phase sera than in sera from healthy individuals (P = 0.002). An antibody response against Clf was most frequent in patients suffering from osteitis plus septic arthritis and from endocarditis (80% positive). The antibody response against Efb appeared to develop later in the course of disease. A possible biological effect of measured antibodies was demonstrated with the help of an inhibition ELISA, in which both high-titer and low-titer sera inhibited the binding of bacteria to fibrinogen. In conclusion, we have demonstrated in vivo production of *S. aureus* fibrinogen binding proteins during deep *S. aureus* infections and a possible diagnostic and prophylactic role of the corresponding serum antibodies in such infections.

The serological diagnosis of serious *Staphylococcus aureus* infections in the routine laboratory presently is based mainly on measuring antibodies against extracellular proteins, such as alpha-toxin or lipase, or against cell wall components, such as peptidoglycan, teichoic acid, or capsular material (4, 7, 8, 19). These antigens have been selected for serological diagnosis partly due to their possible role in bacterial virulence (17, 22). Recently, various surface-associated proteins of *S. aureus* which might contribute to virulence and promote adherence to the host immune system are exposed to them. The presence of *S. aureus* attachment. It has been suggested that this bacterial coating may contribute to the evasion of host defenses (9).

Another important function of fibrinogen is to act as a cellular agglutinin, promoting interaction between several types of cells, such as staphylococci and streptococci, and also with platelets. These interactions involve binding to specific binding proteins on the cells. *S. aureus* cells form macroscopic clumps (clumping) when they are suspended in plasma. This reaction is the result of the avid binding of the dimeric plasma protein fibrinogen to the specific binding protein clumping factor (Clf) on the bacterial cell surface (11). Clf has been shown to be the major cause of *S. aureus* adhesion to fibrinogen (16).

Extracellular fibrinogen binding protein (Efb) is a constitutively produced 15.6-kDa protein and is one of three described fibrinogen binding proteins which are secreted into the medium (2, 21).

With the use of allele replacement mutants in experimental animal infection models, Clf has been shown to be of importance in endocarditis (18) and Efb has shown to play a role in the pathogenesis of wound infection (20). In an experimental mouse mastitis model, immunization with Efb was shown to give protection (15).

The aim of this study was to investigate whether patients with *S. aureus* septicemia produce antibodies against two antigen binding proteins, the cell surface-bound Clf and the extracellular Efb. A demonstrable antibody response would actually indicate that these proteins are produced in vivo and that the host immune system is exposed to them. The presence of an antibody response against these proteins may also add di-
agnostic information when patients with putative invasive S. aureus infection are being evaluated.

MATERIALS AND METHODS

Patients. Forty-one patients with S. aureus septicemia admitted to the Department of Infectious Diseases, Orebro Medical Center Hospital, were included and have been described earlier (28). The clinical diagnosis of S. aureus septicemia was verified by at least two positive blood cultures with the Bactec 660 HP system (Becton Dickinson, Paramus, N.J.). The mean age of the septicemic patients was 65 years (range, 13 to 93 years). Serum samples were collected sequentially (n=108) and stored at -80°C until analysis. Acute-phase samples were drawn 0-8 days and convalescent-phase serum samples were drawn 14 to 30 days after onset of disease. The S. aureus septicemia patients were divided according to complicated infections, as follows: all endocarditis cases (n = 10), osteomyelitis cases except those with endocarditis (n = 8), joint infection and septic arthritis (n = 12), abscesses only (n = 4), and uncomplicated cases (n = 7).

Production of fibrinogen binding proteins. Clf was purified from E. coli XL-1 harboring plasmid pCFS3 (kindly supplied by T. J. Foster, Dublin, Ireland), derived from pQE30 (Qiagen, Basel, Switzerland), expressing a His6 fusion protein. This 42-kDa fusion protein contains residues 221 to 550 of the Clf region that has the fibrinogen binding domain. The His6-Clf fusion protein was purified by using nickel chelator according to the instruction provided by Qiagen. Efb was purified from S. aureus (strain Newman) as described earlier (21). One liter of S. aureus culture was grown overnight at 37°C in Luria-Bertani medium. The culture was centrifuged to cell pellets, and fibrinogen binding proteins in the supernatant were isolated by affinity chromatography on fibrinogen-Sepharose (Pharmacia, Uppsala, Sweden). Bound proteins were eluted with 0.7% acetic acid (pH 5.0) and dialyzed against 40 mM phosphate buffer (pH 6.5), and the different fibrinogen binding proteins were separated from each other by last protein liquid chromatography on a Mono-S column. Elution with a gradient of 0 to 1 M NaCl resulted in three peaks of protein. The N terminus from each peak was sequenced and analyzed: the N-terminus sequence from the first peak corresponded to the coagulase sequence, that from the second peak (0.35 to 0.45 M NaCl) corresponded to Efb, and that from the third peak (0.65 M NaCl) corresponded to Efb. Further identification of the second peak was done by immunoblotting with specific rat anti-Efb (21).

ELISA. Coating concentrations were 2 μg/ml for Clf and 0.6 μg/ml for Efb. The 220-kDa fibrinogen monomer (Bio-Suite, Denmark) was used as the antigen source. The ELISA method has been described earlier (22). The working volume was 100 μl, and after each step the microwells were washed three times with phosphate-buffered saline (pH 7.4) (PBS) plus 0.05% Tween 20 (PBS-T). Briefly, microwells were coated with the appropriate antigen diluted in PBS-T and incubated overnight at 4°C. Serum samples diluted 1/16 in PBS-T were applied and incubated for 1 h at 37°C. Each patient sample was titrated in a twofold dilution (1/500 to 1/16,000). Alkaline phosphatase conjugated to goat anti-human antibodies (Sigma Chemical Co., St. Louis, Mo.) was used at a dilution of 1/500 in PBS-T and was then added, as the reaction was developed in 1 h at 37°C. For every two plates, three control sera (two positive and one negative) and a reference serum (consisting of pooled sera from six patients with confirmed sepsis) were included. The control sera were used in a single dilution in duplicate wells, and the reference serum was titrated like the patient samples. After the final wash, the reaction was developed by the addition of p-nitrophenylphosphate substrate (Sigma Chemical Co.). The enzymatic reaction was measured at 405 nm in a Tittertek Multiscan microplate reader (Flow Laboratories, Irvine, Scotland) after 45 min of incubation for Clf and after 20 min for Efb. The data were transmitted online from the reader to a computer, and calculations were performed with the Unitacle software (PhPlate Stockholms AB, Stockholm, Sweden).

Interpretations of ELISA results. In order to be able to perform comparative calculations with the ELISA results, the obtained absorbance values were transformed into arbitrary units by using the reference line units calculation method (23). The dilution curve of each sample was made parallel to that of the reference serum, after which the two curves were compared. The reference serum was given the value of 1,000 U for both antigens (Unitacle software; PhPlate Stockholm), and the serum antibody levels were expressed in these units. A level of 2,000 U in a sample thus means that this sample could be diluted to twice the dilution of the reference serum, as compared with the dilution curves. The lowest level that did not indude in the calculations was 60 U. The upper limit was used for normal antibody levels was established with the healthy controls. The upper cutoff level was set at the upper 95th percentile. Levels above this cutoff level to be included in the calculations was 60 U. Further identification of the second peak was done by immunoblotting with specific rat anti-Efb (21).

RESULTS

Antibodies against Clf and Efb in a healthy control population. Antibody levels against Clf and Efb in a healthy control population (n = 72) were estimated (Fig. 1). No age-correlated variation was found, and therefore a common upper cutoff limit was used without age corrections. The upper limit, corresponding to the 95th percentile, was 440 U for Clf and 750 U for Efb. No correlation between the Clf and Efb antibody levels was established with the healthy controls. The upper cutoff level, cor-
these all but 3 (63%) showed a significant rise in antibody levels against at least one of the antigens. One-third of the patients were already positive in their acute-phase sample. Most patients increased their antibody levels to both antigens, as indicated by the statistically significant correlation coefficient for the levels in the convalescent-phase sera (r = 0.38; P < 0.02) (Fig. 2C).

Antibody response in relation to type of infection. The serological response against Clf was higher and occurred earlier than the response against Efb. About 80% of both patients with osteitis plus septic arthritis and those with endocarditis were positive (Fig. 4). The greatest increase in the percentage of positive patients was found in those with bone and joint infections (0 to 75%) (Fig. 4).

Inhibition of bacterial binding to fibrinogen. Bacteria were bound to immobilized fibrinogen with and without preincubation with patient sera, and binding was detected by the same sera. It was evident that both high-titer and low-titer sera inhibited the binding to fibrinogen to various extents (Fig. 5), thus indicating a possible biological effect of the measured antibody levels.

**DISCUSSION**

Surface-associated proteins of *S. aureus* and their possible roles as virulence factors have been studied with various animal models (15, 18, 20). However, knowledge about the serological response against these proteins in patients suffering from deep *S. aureus* infections was lacking (3). Studies on antibody levels against potential vaccine candidates in health and disease are important for the development of an effective multicomponent vaccine against deep infections caused by *S. aureus* (14). In the present study we analyzed the serum immunoglobulin G (IgG) responses against two fibrinogen binding proteins of *S. aureus*: Clf, which is expressed mainly on the bacterial surface, and Efb, which is an extracellular protein.

In a healthy population we found the same pattern as with other *S. aureus* antigens, namely, that healthy individuals show highly variable levels (100-fold variation) of serum antibodies against the studied antigens (Fig. 1), but all individuals had detectable IgG levels. Similar results are found with other staphylococcal antigens, such as alpha-toxin (8, 10), lipase (26), enterotoxins (12), toxic shock syndrome (TSS) toxin (1), teichoic acid (8, 10), and peptidoglycan (4), which indicates previous experience of staphylococcal infections and thereby antibodies raised against these antigens in the population. The presence of detectable antibody levels, both in the normal population and in patients, clearly indicates that these two fibrinogen binding proteins are expressed by *S. aureus* in vivo.

In most of the previous studies on antibody responses to various *S. aureus* antigens, the antibody levels have been expressed as absorbances (titers) in a one-dilution indirect ELISA (8, 10), thus making interlaboratory comparisons and meaningful calculations difficult. In the present study, each serum was titrated in six twofold dilutions, and correlation curves were calculated. They were in turn referred to results for a standard serum given arbitrary units (23). This method facilitates interlaboratory comparisons when the same standard serum is used. Furthermore, the units obtained represent true titers; i.e., they indicate how many times the tested serum can be diluted in order to give the same curve as the standard serum. Such titers are “calculable”; i.e., a doubling of units between acute- and convalescent-phase sera actually indicates that the convalescent-phase serum can be diluted one twofold dilution step further than the acute-phase serum. This can not be stated for absorbance values.

The poor correlation between the IgG levels against Clf and Efb in the normal population (Fig. 2) indicates that the mea-
sured antibodies were independently produced, and thus these two antigens could give us complementary diagnostic serological information. In fact, with alpha-toxin and teichoic acid as antigens with the patients' sera, 73% of the patients were deemed serologically positive (8), and the use of Clf and Efb as antigens resulted in 71% of the patients being deemed positive. Accordingly, combining all four antigens raised the percentage of positive patients to 88% (data not shown).

The kinetics of the antibody responses against the two studied antigens were different. Clf showed the expected kinetics, with a rapid onset of antibody production, seen as high levels in the acute-phase sera, and an expected further rise in antibody levels during the disease. In contrast, antibody levels to Efb were significantly lower in the acute-phase sera than in the normal population, and only four patients, who were suffering from endocarditis, showed serum IgG levels above the cutoff level (Fig. 3 and 4). Although the IgG levels increased during disease, the average level in the convalescent-phase sera still barely reached that of the normal population (Fig. 3).

These differences might be due to a weaker stimulation of the immune system by the Efb antigen than by the Clf antigen, but this explanation seems less probable since the antigens appear to induce similar levels of antibodies in the normal population. Alternatively, the formed antibodies may form complexes with freely circulating antigen. This complex formation may cause a consumption of serum antibodies, rendering them not detectable in the ordinary ELISA. This consumption might be more pronounced for the extracellularly released Efb than for the cell-bound Clf, a phenomenon which would result

FIG. 2. Comparisons of antibody levels against Clf and Efb in a healthy control population (A), in acute-phase samples from septicemic patients (B), and in convalescent-phase samples from the same patients (C). Dashed lines indicate upper 95th percentiles.

FIG. 3. Box plots of antibody levels against Clf (top) and Efb (bottom) in healthy and septicemic individuals. The box comprises 50% of the individuals, and the vertical lines comprise the individuals within the area representing twice that of the box height. The horizontal line in each box represents the median value.
in the observed differences in antibody levels during an active infection. However, the difference may also reflect the fact that individuals with a weaker antibody production against Efb are more prone to develop deep (or serious) staphylococcal infections. Such a possible relation between infection susceptibility and antibody formation was reported earlier for *S. aureus* alpha-toxin (8), and a recognized correlation between low antibody levels and disease susceptibility exists for TSS toxin and TSS (1, 5). The polysaccharide capsules of certain *S. aureus* strains have been implicated as a virulence factor that is neutralized by specific antibodies (13).

The earlier response against Clf than against Efb during the course of disease reminds one of the fact that Clf may be produced to anchor the cell to fibrinogen (and fibrin) deposits, probably early in the infectious phase, while Efb is constitutively produced and reaches larger amounts at a later phase, probably inhibiting attachment by Clf and thereby facilitating the spread of bacteria (22). The most probable reason is that
patients with lower antibody levels, whether or not they reflect a decreased ability to produce the particular antibodies, are at a higher risk to develop a deep infection with *S. aureus* (14).

Although the number of samples is too small for division into subgroups, Fig. 4 indicates that certain complications give rise to a more pronounced antibody response than others. Uncomplicated sepsis was shown by Colque-Navarro and co-workers, using the same patients, to give rise to comparatively higher antibody titers against alpha-toxin and teichoic acid (8), but against the fibrinogen binding antigen, the response was opposite. Instead, patients suffering from osteitis in connection with arthritis and patients with endocarditis showed the most evident serological response (83 and 80%, respectively, with both antigens). Patients with osteitis only and with abscesses showed a weaker response (63 and 50%, respectively), which is in concordance with earlier observations on other antigens (10).

Thus, this study has shown that the two fibrinogen binding proteins Clf and Efb are produced in vivo, that antibodies against them are present to various extents in the normal population, and that increased levels are achieved upon *S. aureus* infections in many cases. During the disease, such an increase in antibody levels is seen earlier for Clf than for Efb. In fact, acute-phase sera from patients show lower mean IgG levels against Efb than in the normal population. The patient sera inhibited the binding of *S. aureus* bacterial cells to immobilized fibrinogen in parallel with the measured IgG levels, a finding which indicates a possible biological effect of these antibodies. Furthermore, the antibodies against Clf were measured against a recombinant molecule representing mainly the fibrinogen binding domain. The studies indicate that Clf and Efb should be considered in future work with immunoprophylactic tools against deep infections with *S. aureus*. The opsonizing effect of IgG against cell-bound antigens is considered to be of outmost importance in the host defense against *S. aureus* infections (14), and it is conceivable that antibodies against cell-bound fibrinogen binding proteins, like Clf, would act as such.

**REFERENCES**