Serotyping and Electron Microscopy Studies of *Staphylococcus aureus* Clinical Isolates with Monoclonal Antibodies to Capsular Polysaccharide Types 5 and 8

H. K. HOCHKEPPEL,^{1*} D. G. BRAUN,¹ W. VISCHER,¹ A. IMM,¹ S. SUTTER,¹ U. STAEUBLI,¹
R. GUGGENHEIM,² E. L. KAPLAN,³ A. BOUTONNIER,⁴ AND J. M. FOURNIER⁴

Pharmaceutical Research Laboratories, CIBA-GEIGY Limited, CH-4002 Basel,¹ and Geologisch paläontologisches Institut, University Basel, CH-4056 Basel,² Switzerland; Department of Pediatrics, University of Minnesota Medical School, Minneapolis, Minnesota 55455³; and Institut Pasteur, F-75724 Paris Cedex 15, France⁴

Received 22 August 1986/Accepted 19 November 1986

Monoclonal antibodies to *Staphylococcus aureus* capsular polysaccharide types 5 and 8 were prepared and used to serotype 821 clinical isolates of *S. aureus* from four countries. The capsular polysaccharide-binding sites on the bacterial membrane were examined by transmission and scanning electron microscopy.

The currently available data about the cell surface components thought to be associated with the virulence of *Staphylococcus aureus* are limited and still partly confusing. This fact is in strong contrast to the frequent occurrence of serious and lethal staphylococcal infections all over the world. The involvement of capsular polysaccharides (CP) in the invasiveness of many bacteria, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Escherichia coli*, and group B streptococci, is well established (14, 15). CP have been identified in clinical isolates of *S. aureus*, and a method for the serological typing of the CP has been proposed (5). A survey of 295 isolates showed the predominance of types 5 and 8 CP (1). This predominance was confirmed in another survey (18). In those studies, however, they used polyclonal rabbit antisera.

Monoclonal antibodies (MAb) reactive with types 5 and 8 CP have been described, but their usefulness in characterizing *S. aureus* clinical isolates has not yet been determined (11). We report here the use of a series of types 5- and 8-specific MAb as diagnostic tools for the serotyping of 821 *S. aureus* clinical isolates from four countries in Europe and the United States. These MAb were also used to study, by transmission and scanning electron microscopy, the capsule of *S. aureus* under different conditions for bacterial growth.

MATERIALS AND METHODS

Bacteria. Clinical isolates of *S. aureus* were collected from four different countries: Basel, Geneva, and Zurich in Switzerland; Freiburg in the Federal Republic of Germany; Lyon and Paris in France; and Minneapolis in the United States. These strains were all isolated from clinical infections in humans either from blood cultures or from other purulent or inflammatory processes.

Production of MAb. Female BALB/c or C57 BL/6 mice were immunized four times with 10^8 heat-inactivated cells of *S. aureus* standard strains of serotypes 5 (phage type 6/42E/47/54**) and 8 (phage type 29/52/ 52A/80*) in Freund adjuvant, and spleen cells of these mice were then fused with SP2/0 mouse myeloma cells (16) by the method of G. Köhler and C. Milstein (9). Hybridoma cells were cloned by limiting dilutions in microtiter plates, and supernatants were screened by agglutination with *S. aureus* reference strains of

known serotypes and phage lysotypes. Established MAb were checked for CP specificity by double diffusion against purified *S. aureus* CP of serotypes 5 and 8 (2); all MAb were polysaccharide as well as serotype specific. There was no agglutination cross-reaction of MAb with the other *S. aureus* serotype. The immunoglobulin isotypes of the various MAb were determined by the Ouchterlony method. All experiments described were performed with the following MAb (isotype, serotype, agglutinating titer): 814 S 15.4 (μ , 5, 1:5,120), 825 S 23.1 (μ , 5, 1:2,560), 831 S 15.2 (γ 3, 5, 1:1,280), 831 S 15.14 (γ 3, 5, 1:1,280), 813 S 16.3 (μ , 8, 1:1,280), 828 S 96.22 (γ 3, 8, 1:1,280), 828 S 96.3 (γ 3, 8, 1:1,280), and 829 S 64.3 (μ , 8, 1:2,560).

A competition enzyme-linked immunosorbent assay to define common or different epitope specificity of MAb to *S. aureus* was performed in microtiter assay plates (Dynatech Laboratories, Inc.). A sample (50 μ l) of a killed *S. aureus* suspension containing 10^8 cells per ml was added to each well, the plate was centrifuged for 10 min at 2,000 rpm ($400 \times g$), and the cells were fixed to the microtiter wells with 1% glutaraldehyde for 30 min at 37°C. The wells were washed with phosphate-buffered saline (PBS) and blocked with bovine serum albumin (1% bovine serum albumin in PBS). Samples of each MAb to *S. aureus* were coupled to alkaline phosphatase, and the binding of labeled antibodies to *S. aureus* was competed with different dilutions of nonlabeled MAb.

Serotyping of *S. aureus* with MAb by agglutination. *S. aureus* cells were grown on Columbia broth medium (no. 0944; Difco Laboratories, Detroit, Mich.) containing 4% NaCl and 4% agar (BBL Microbiology Systems, Cockeysville, Md.) per liter. Cultures were grown for 20 h in 5% CO₂ at 37°C. This allows optimal expression of the bacterial capsule (2). Bacteria were harvested from the plate by washing with 0.01 M Tris hydrochloride buffer (pH 7.2) and then adjusted to a density of McFarland 10, corresponding to approximately 3×10^9 live bacteria. Suspensions of 10^9 bacterial cells per ml were heat killed for 30 min at 100°C and then sonicated for 1 min just before testing. For agglutination 25 μ l of the staphylococcal suspension was mixed with 50 μ l of MAb solution (in PBS) in wells of Costar 96-well microtiter plates. For the titration MAb were diluted in PBS. The reaction mixture was incubated at room temperature with

* Corresponding author.

FIG. 1. Serotyping of *S. aureus* by RIE. Samples (5 μ l) of purified type 5 CP (16 μ g/ml in well 1, 8 μ g/ml in well 2, 4 μ g/ml in well 3), purified type 8 CP (16 μ g/ml in well 4, 8 μ g/ml in well 5, 4 μ g/ml in well 6), and supernatants of autoclaved bacteria (wells 7 through 16) were placed in 1% agarose gel containing anti-type 5 MAb (A), or anti-type 8 MAb (B) and subjected to electrophoresis (15 V/cm, 4°C, 1 h). Wells 8, 10, and 12 contained type 5 bacteria; wells 7, 9, 11, 14, and 16 contained type 8 bacteria; wells 13 and 15 contained bacteria that were neither type 5 nor 8.

gentle shaking for 10 min, and the bacterial samples were then checked for agglutination.

Serotyping of bacteria by RIE. Bacteria were grown overnight at 37°C on Columbia medium agar slants. Bacterial cells were suspended in 2 ml of PBS (pH 7) and autoclaved at 121°C for 60 min. After centrifugation, the supernatant was retained and stored at -20°C. Rocket immunoelectrophoresis (RIE) was carried out on Gelbond film (60 by 125 mm; Marine Colloids, Rockport, Maine) covered with 1% agarose (Indubiose A 37 HAA; Reactifs IBF, 92390 Villeuevela-Garrenne, France) in 0.025 M barbital-acetate buffer (pH 8.6) containing 35 μ g of MAb per ml. Samples of supernatants of autoclaved bacteria (5 μ l) were subjected to electrophoresis (15 V/cm, 4°C, 1 h) in 0.05 M barbital-acetate buffer (pH 8.6). A sample of purified CP served as standard.

Transmission electron microscopy. *S. aureus* organisms with low or high CP content were obtained by varying the culture conditions. Low-CP-containing bacteria were cultured on Columbia agar slants with airtight caps, whereas high-CP-containing bacteria were grown on slants with loose caps permitting good air exchange. Immunological quantitation of CP was performed by RIE. Purified CP served as standard. The amount of CP in the bacterial membranes was expressed by the ratio of CP (micrograms per milliliter) to protein (milligrams per milliliter) in the supernatant of autoclave extracts. *S. aureus* cells were agglutinated with MAb and then prepared for transmission electron microscopy by double fixation by the method of Geuze et al. (3), dehydrated in acetone (increasing from 50 to 100%), and then embedded in Epon (10). The embedded samples were cut and then stained with 20-nm gold particles (200 Å) as well as with lead citrate (13) and finally examined with a Phillips 300 transmission electron microscope.

Scanning electron microscopy. Live *S. aureus* cell preparations were washed in PBS without calcium and magnesium and then fixed on glass cover slides with 5% glutaraldehyde. Fixed staphylococci were then incubated with serotype 5- or 8-specific MAb for 2 h at room temperature. After washing, the slides were incubated with gold-labeled goat anti-mouse immunoglobulin (40-nm gold particles; Janssen) in Tris-NaCl (pH 8.2) containing 0.1% bovine serum albumin. After 1 h, an additional fixation was carried out with 2.5% glutaraldehyde. The sample was dehydrated in 100% acetone and then dried overnight by critical-point drying with liquid CO₂. The dried samples were spattered with 20-nm gold particles and visualized in a Raster electron microscope (Philips 515).

RESULTS

Characterization of MAb. Twenty MAb of either isotype μ or γ_3 were obtained, and their reactivity with purified *S. aureus* CP was demonstrated by double diffusion and RIE (Fig. 1). The agglutination titer of the individual MAb varied from 1:1,280 to 1:5,120. Four type 5-specific MAb and three type 8-specific MAb were tested for binding to homogeneous or heterogeneous CP epitopes on *S. aureus* in a competition enzyme-linked immunosorbent assay in which *S. aureus* cells were fixed to the assay plate by glutaraldehyde (see Materials and Methods). All type 5-specific MAb possessed different epitopes, whereas all type 8-specific MAb recognized the same epitope. Whether this indicates higher heterogeneity in the composition of *S. aureus* type 5 CP has still to be demonstrated.

Serotyping of *S. aureus* clinical isolates with MAb. To show the applicability of anti-*S. aureus* MAb for routine characterization of clinical isolates, studies with the MAb listed in Materials and Methods were performed with 821 *S. aureus* isolates from four different countries by agglutination or RIE. For comparison, these isolates were also phage typed at the Phage Typing Center, University of Bonn, Bonn, Federal Republic of Germany, correlating with the serological typing (manuscript in preparation).

These MAb were able to type at least 80% of all tested clinical isolates (Table 1); 26% were serotype 5, 55% were serotype 8, and only 19% were nontypable. No significant difference was found in the serotype distribution pattern between the individual countries, and the predominance of serotypes 5 and 8 among clinical isolates, observed already by others (1), was confirmed. There were no cross-reactions between serotypes 5- and 8-specific MAb.

Comparison of the two serotyping methods. Serotyping of 97 *S. aureus* strains of the same bacterial culture by agglutination and by RIE resulted in an excellent correlation of the two methods (Table 2). However, when 112 *S. aureus* strains of two different cultures were tested, the RIE typing was more sensitive (8 nontypable strains by RIE compared

TABLE 1. Serotyping of clinical *S. aureus* isolates (encapsulated) with MAb

Origin of strain	Type 5, no. (%)	Type 8, no. (%)	Other type, no. (%)	Total, no. (%)
Switzerland (Basel, Geneva, Zurich)	69 (35)	90 (45)	40 (20)	199 (100)
Federal Republic of Germany (Freiburg)	26 (11)	158 (67)	53 (22)	237 (100)
United States (Minneapolis)	20 (36)	32 (59)	3 (5)	55 (100)
France (Lyon, Paris, Tours)	96 (29)	170 (52)	64 (19)	330 (100)
All	211 (26)	450 (55)	160 (19)	821 (100)

TABLE 2. Comparison of agglutination and RIE serotyping methods

Bacteria from ^a :	No. of isolates (%) with the following results			
	Same result by both	Different results	Typable by RIE only	Typable by agglutination only
Same culture	82 (73)	0 (0)	22 (20)	8 (7)
Different cultures	89 (92)	2 (2)	6 (6)	0 (0)

^a A total of 97 clinical isolates were tested by both methods with samples from the same culture; 112 clinical isolates from different cultures were also tested by both methods.

with 22 nontypable strains by agglutination) (Table 2). The difference in sensitivity in the latter case is probably due to variability in encapsulation of *S. aureus* from culture to culture, even for the same strain.

Electron microscopy study. To localize the CP epitopes on the bacterial cell wall, we performed a series of transmission and screening electron microscopy experiments with the MAb which had been used before the serotyping. Figures 2 and 3 show gold-stained transmission electron microscopy pictures of MAb agglutinated with staphylococcus preparations harvested in a quiescent, nondividing phase containing a small amount of cell wall CP (Fig. 2) as compared with

FIG. 2. Transmission electron microscopy of *S. aureus* after embedding, cutting, agglutination with MAb, and staining with gold-labeled goat anti-mouse immunoglobulin antibodies. Bacteria contained small amounts of CP (type 5, 1 μ g of CP per mg of protein; type 8, 10 μ g of CP per mg of protein). (A) Staphylococcus serotype 8, MAb serotype 8 specific; (B) staphylococcus serotype 5, MAb serotype 5 specific; (C) staphylococcus serotype 8, MAb serotype 5 specific; (D) staphylococcus serotype 5, MAb serotype 8 specific.

FIG. 3. Transmission electron microscopy of bacteria containing high amounts of CP (type 5, 5 μ g of CP per mg of protein; type 8, 30 μ g of CP per mg of protein). (A) Staphylococcus serotype 8, MAb serotype 8 specific; (B) staphylococcus serotype 5, MAb serotype 5 specific; (C) staphylococcus serotype 8, MAb serotype 5 specific; (D) staphylococcus serotype 5, MAb serotype 8 specific.

MAb reacted with staphylococci in the division phase with a large amount of cell wall CP (Fig. 3). The transmission electron microscopy studies clearly demonstrated that, depending on the growth conditions applied for *S. aureus*, the distribution of CP in the bacterial cell wall was significantly different. Whereas low-CP-containing bacteria bound the MAb at the cell wall in patches (Fig. 2), high-CP-containing bacteria had the CP-binding epitopes only in hairlike zones surrounding the bacterial cell wall (Fig. 3), indicating that dividing bacteria might protect themselves against the immune system of the host by producing or even secreting more CP, neutralizing or keeping off the host's antibodies.

Scanning electron microscopy. Back-scattering scanning electron microscopy indicated that the CP epitopes of the bacterial membrane of low-CP-containing staphylococci to which MAb bound were not uniformly distributed over the whole surface, but rather were located on separate membrane areas (Fig. 4).

DISCUSSION

S. aureus was and still is an important bacterial pathogen (14, 17). It is the most common source of serious soft tissue infections and bacteremia. One of the most dramatic syndromes caused by *S. aureus* after bacteremia is acute

FIG. 4. Scanning electron microscopy of *S. aureus* serotype 8 bacteria agglutinated with serotype 8-specific MAb by the back-scattering technique. Staining was with gold-labeled goat anti-mouse immunoglobulin antibodies. (A) Total view; (B) section.

bacterial endocarditis leading to septic shock or disseminated intravascular coagulation. The mortality rate in such cases ranges from 40 to 80%, depending on the age of the infected person. Frequent complications include high fever, pulmonary abscesses, meningitis, glomerulonephritis, specific arthritis, etc. Clinical isolates of *S. aureus* have been classified into eight types on the basis of their CP (6). Predominantly two CP types, 5 and 8, occur among clinical isolates (1), accounting for about 70% of all isolates. These results were based on polyclonal typing sera, obtained from rabbits, produced against eight prototype strains (6). The high prevalence of these two CP types in clinical bacteremic isolates suggests the importance of this capsular antigen in staphylococcal disease. The type 8 CP of *S. aureus* has been purified and characterized (2). Karakawa et al. (5) have described a method for serological typing of CP by using the above-mentioned rabbit antisera. Nelles et al. (11) first described type-specific MAb to clinical *S. aureus* isolates of types 5 and 8. However, the anti-type 5 MAb did not precipitate purified CP. Here we describe a panel of MAb to *S. aureus* types 5 and 8 of immunoglobulin isotypes μ and $\gamma 3$ which specifically do precipitate purified type 5 or 8 CP and also agglutinate whole bacteria. The restriction of carbohydrate-specific MAb to immunoglobulin isotypes μ and $\gamma 3$ has been observed previously (4, 12).

Using these MAb, we have now serotyped 821 clinical isolates from four different countries by agglutination and RIE, and thus we have demonstrated the relatively easy and reliable applicability of these MAb for the characterization of clinical isolates, identifying most of the strains (a prospective study will finally determine this), whereas at the present time the phage typing method is still cumbersome and often not available. The typing results of both methods agreed very well, but the RIE was more sensitive. Our serotyping results confirmed again the predominance of types 5 and 8, and the distribution of types 5 and 8 were similar in the United States and Europe. Of the total clinical isolates examined, 80% were characterized by our MAb as either type 5 or 8, and no cross-reaction between type 5- and type 8-specific MAb was observed. This is in agreement with the work of Nelles et al. (11) and Sompolinski et al. (18) showing that at least 70% of *S. aureus* bacteremia isolates observed belonged to serotype 5 or 8. Although the epidemiological

application of the *S. aureus* CP-specific MAb might be limited, the MAb may be useful diagnostic tools in clinics, for example, for the fast diagnosis of deep-seated infections by direct detection of CP released into body fluids. The sensitive RIE typing method allows the quantitative determination of types 5 and 8 CP and accounts for the 80% typability of *S. aureus* clinical isolates in comparison to the 70% typability reported by others (11, 18). Certainly the percentage of typable clinical isolates with RIE will be even further improved by preparing MAb to other capsular types. Sompolinski et al. (18) have performed morphological electron microscopy studies with anti-type 8 CP MAb bound to the bacterial membrane of *S. aureus*. In this report we describe electron microscopic binding experiments of CP-precipitating MAb with *S. aureus* isolates of which the CP content had been quantitatively determined by RIE. The specificity of binding reactions of the individual MAb to type 8 or 5 clinical isolates was ensured by also incubating these isolates in a parallel reaction with MAb specific for the other serotype. These binding studies clearly reveal the variation in the degree of bacterial encapsulation depending on the culture conditions (Fig. 2 and 3). Low-CP-containing bacteria bind MAb at the bacterial membrane in patches, whereas high-CP-containing bacteria bind MAb in hairlike zones surrounding the cell wall. One could imagine that the same holds for the in vivo situation, and this may correlate very well with the antiphagocytic function of CP as described by Karakawa et al. (6–8). For example, in animal infection models with pneumococci, in vitro studies with complement and analyses of disease isolates from patients revealed a direct correlation between the virulence or invasiveness of a strain and the concentration of CP (14). Therefore, such MAb not only may be useful diagnostic tools, but also may be applicable for therapy.

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