Characterisation and functional aspects of monoclonal antibodies specific for surface proteins of coagulase-negative staphylococci

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Summary. Mouse monoclonal antibodies (MAbs) raised against whole cells of *Staphylococcus epidermidis* strain 354 were characterised morphologically and functionally. Nine MAbs showed strong reactivity with coagulase-negative staphylococci (CNS). Only two MAbs were specific for CNS; both belonged to the IgG₁ subclass, and one, MAb 36.4, reacted only with the strain used for immunisation. In immunoblotting, both CNS-specific MAbs 36.3 and 36.4 reacted strongly with cell-wall protein bands of 220 Kda of *S. epidermidis* strain 354 and weak reactivity was observed with a 110-Kda band. MAb 36.3 reacted also with 220–230 Kda bands of two other *S. epidermidis* strains (291 and ATCC 35984) and a 160–180 Kda band of *S. epidermidis* strain 354. Only MAb 36.4 promoted phagocytosis of strain 354 by polymorphonuclear leucocytes (PMNL) and monocytes, whereas MAb 36.3 and the other MAbs lacked this activity. Opsonisation of *S. epidermidis* with MAb 36.4 in the presence of complement enhanced uptake by PMNL, but not by monocytes. Furthermore, *S. epidermidis* strain 354 opsonised with MAb 36.4 induced chemiluminescence of PMNL. Immuno-gold electronmicroscopy with both MAbs 36.3 and 36.4 demonstrated a homogeneous distribution of gold particles on the surface as well as close to the surface of *S. epidermidis*.

Introduction

Coagulase-negative staphylococci (CNS), particularly strains of *Staphylococcus epidermidis*, are increasingly recognised in infections related to foreign bodies such as intravascular^{1–3} and peritoneal dialysis catheters,^{4,5} prosthetic joints and heart valves.⁶ Pathogenesis of infections related to such foreign bodies is still unclear although surface hydrophobicity and the production of extracellular slime are probably involved in adherence of CNS to biomaterials. Treatment of CNS with proteolytic enzymes resulted in reduced adherence to catheter segments and decreased hydrophobicity, suggesting a role for hydrophobic surface-exposed proteins in adherence.⁷ Moreover, recent evidence indicates that surface hydrophobicity of CNS is important in complement-dependent opsonisation.⁸ Both of these results suggested an important role for the hydrophobic parts of exposed proteins of CNS.

In the studies reported here we have characterised two monoclonal antibodies (MAbs) specific for surface proteins of CNS. We have examined opsonisation of

one *S. epidermidis* strain by a MAb, as measured by the ability to elicit chemiluminescence from human polymorphonuclear (PMNL) and monocytes, and the reactivity of MAbs with surface structures of strains of *S. epidermidis* by immuno-gold electronmicroscopy (EM).

Materials and methods

Bacterial strains

Forty-three CNS isolates (*S. epidermidis*) from patients with catheter-related bacteremia, two CNS strains from the American Type Culture Collection (*S. epidermidis* ATCC 35984 and *S. hominis* ATCC 35982), two other strains of *S. hominis* and six strains of *S. aureus* (Cowan I, EMS, Wood 46, H+, H–, no. 304)⁹ were used in our experiments. All strains were lyophilised and stored at –70°C.

For specificity tests, 10 strains of enterobacteria (*Acinetobacter anitratus*, *Citrobacter diversus*, *C. freundii*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *P. vulgaris*, *Serratia marcescens*) were used.

Monoclonal antibodies

MABs were raised against a clinical isolate of *S. epidermidis* (strain 354) by the method of Kohler and Milstein¹⁰ with minor modifications. Briefly BALB/c mice were immunised intraperitoneally (i.p.) with 200 µl of a suspension of live cells (5×10^7 cfu) in four injections. An intravenous booster dose of 200 µl (5×10^7 cfu) of the suspension was given 3 days before the fusion. Spleen cells were fused with cells from a mouse myeloma cell line, SP 2/0-Ag 14¹² in a ratio of 5:1. The hybridomas were screened for antibody activity against intact cells of strains of *S. epidermidis* by ELISA; 96-well microtitration plates were coated overnight in phosphate-buffered saline (PBS, pH 7.4) with bacteria. For production of ascites, selected hybridomas (5×10^5 cells) were injected i.p. into pristane-(Janssen Chemica, Beerse, Belgium)-primed BALB/c mice. MABs were purified by high performance liquid chromatography (HPLC) on an ABx-column (J. T. Baker Chemical Company, Philipsburg, NJ, USA). Cross-reactivity was determined by ELISA with *S. hominis*, *S. aureus* and enterobacterial strains. An OD value ≥ 0.5 was considered to be a positive result.

Preparation of staphylococcal proteins

Bacteria were grown overnight at 37°C in Nutrient Broth (Difco). Cells were washed in PBS (pH 7.4) and harvested by centrifugation at 3000 g for 15 min. Bacteria were resuspended in 1 ml of PBS containing 100 µg of lysostaphin (Sigma) and incubated at 37°C for 4 h in a rotary mixer (24 rpm). To inhibit proteolytic activity from enzymes released by the bacteria during lysostaphin treatment, phenylmethylsulphonyl-fluoride (PMSF; Sigma) was added to give a final concentration of 1 mM. After centrifugation at 14 000 g for 15 min the supernate containing the staphylococcal polypeptides was held at -20°C in siliconised eppendorf tubes. Protein concentration was measured by Bradford's method.¹³ The sample was lyophilised and adjusted to a protein concentration of 300 µg/ml for further tests.

SDS-PAGE and immunoblotting

After lyophilisation, staphylococcal proteins were dissolved in sample buffer—sodium dodecyl sulphate (SDS) 4% w/v, dithiothreitol 5% w/v, glycerol 40% w/v, and bromophenol blue in 1 M Tris/HCl buffer—and boiled for 5 min; 10 µg of protein per 30 µl was applied to each well for electrophoresis in a polyacrylamide 12% running gel with a 4% stacking gel according to the method of Laemmli.¹⁴ The gel was stained with Coomassie Brilliant Blue R 250 (CBB; Fluka Chemicals).

For immunoblotting staphylococcal proteins were separated in a polyacrylamide 7% gel and transferred from the gel on to nitrocellulose paper (pore diameter

0.45 µm; Schleicher and Schuell, Dassel, Germany). Free protein-binding sites on the nitrocellulose were saturated by incubation overnight in blocking buffer containing Tween 20 0.10% and gelatine 3% in PBS at 4°C. After a further 1 h at 37°C and washing three times in PBS-Tween 20 0.1%, the nitrocellulose paper was cut into strips. Each strip was incubated at 37°C for 1.5 h in 2 ml of MAb 36.4 (1 in 40 000), MAb 36.3 (1 in 5000) or rabbit hyperimmune serum (1 in 1000) in PBS. After washing three times in PBS, the strips were incubated at 37°C for 1.5 h in 2 ml of peroxidase-conjugated affinity purified goat anti-mouse IgG antibody (Cappel, n 3611-0081) (1 in 2500) or anti-rabbit IgG antibody (1 in 5000) in PBS. After washing four times, each strip was incubated in 2 ml of substrate solution—3',3',5',5'-tetramethylbenzidine (Sigma T-2885) 0.2% w/v and sodium dioctylsulphosuccinate (Serva 20540) 0.8% w/v dissolved in 10 ml of ethanol 96% in a water-bath at 56°C was mixed with 30 ml of buffer (pH 5) containing 100 mM citric acid and 200 mM disodium hydrogen phosphate dihydrate; 20 µl of H₂O₂ 30% was added just before use. The reaction was stopped by rinsing the nitrocellulose strip in tap-water.

Immuno-gold labelling for electronmicroscopy

Immuno-gold labelling was performed as described by Voorhout *et al.*¹⁵ *S. epidermidis* cells were fixed in growth medium by adding an equal volume of glutaraldehyde 0.5%, paraformaldehyde 2% in PBS, pH 7.4, and left for 1 h at room temperature. The cells were centrifuged at 2000 g for 10 min, washed with 50 mM glycine in PBS (PBS/Gly) and a solution of BSA 0.5% and gelatin 0.1% in PBS (PBG) for 1 h each. After centrifugation, 50 µl of the cell pellet was resuspended in 450 µl of PBG and incubated with MABs for 1 h at room temperature or overnight at 4°C. Then the cells were rinsed five times with PBG (10 min each) and incubated with 20 µl of swine anti-mouse (SWAM) secondary antibody (Nordic, Tilburg, The Netherlands) 10 mg/ml. Finally the cells were incubated under saturating conditions for 1 h with protein A-gold (12 nm diameter) diluted to OD₅₂₀ 1.0. The incubated cells were rinsed thoroughly five times with PBG, four times with distilled water and then attached to a nickle grid covered with a carbon coated formvar film. After rinsing and drying, the cells were examined with a Philips EM-420 electronmicroscope at 80 kV.

Isolation of human PMNL and monocytes

Human PMNL were isolated from heparinised blood by dextran sedimentation and Ficoll-Paque (Pharmacia, Sweden) density gradient centrifugation. Residual red-blood cells were lysed by hypotonic shock. The resulting PMNL were washed once and suspended in Hanks's Balanced Salts Solution (HBSS)

supplemented with gelatin (HBSS-gel). Mononuclear cells were isolated by the method of Böyum.¹⁶

Phagocytosis assay

Bacteria were radioactively labelled by overnight incubation in Mueller-Hinton Broth (Difco) containing methyl-³[H] thymidine (specific activity 5.0 Ci/mmol; Radiochemical Centre, Amersham) 2 µCi/ml. Opsonisation and phagocytosis assays were performed as described previously.^{17,18}

Opsonins

Human pooled serum (HPS) was obtained from 20 healthy adult donors and stored in 1-ml volumes at -70°C. The source of complement was serum from a child with agammaglobulinaemia with a normal total haemolytic complement activity of the classical and alternative pathways (IgG < 0.01 g/L).

Chemiluminescence assay

Luminol-dependent chemiluminescence (CL) was monitored with an automatic Packard Picolite Luminometer (Packard Instruments, Brussels, Belgium). Opsonised *S. epidermidis* cells (2.5×10^8 /ml) were mixed with luminol (final concentration 1×10^{-5} M) and incubated with 10^7 PMNL/ml in a total volume of 2 ml of HBSS-gel. Controls contained PMNL without bacteria. Experiments were performed at 37°C in the dark and counts were recorded for 6 s over a 30-min period.

Statistical analysis

The differences between sets of results were assessed by Student's *t* test; differences were considered to be significant when $p \leq 0.05$.

Results

Reactivity of MAbs with intact bacteria

MAbs raised in three separate fusions were tested for reactivity with several gram-positive and gram-negative bacterial species. The results of the screening are shown in the table. Nine MAbs were selected that reacted strongly with intact cells of CNS. MAbs of the IgM-class (nos. 17.15, 23.23 and 36.2) showed cross-reactivity with *S. aureus* strains; MAbs 17.15 and 36.2 also reacted with several gram-negative species. None of the IgG MAbs cross-reacted with any gram-negative bacteria. MAb 36.3 subclass IgG₁, reacted with most (28 of 44) of the CNS strains, whereas MAb 36.4 was highly specific for *S. epidermidis* strain 354 used for immunisation and did not show any reactivity with other CNS strains.

SDS-PAGE and immunoblotting

SDS-PAGE of *S. epidermidis* (n = 6), *S. hominis* (2), and *S. aureus* (3) strains showed multiple (25–35) bands 21–230 Kda, with a clustering of bands between 40 and 100 Kda (fig. 1). Clear differences between the various *S. epidermidis* strains are apparent, either in the number of bands, their position in the gel or in the intensity of one or more bands. However, immunoblot analysis of nine CNS strains with rabbit hyperimmune serum, showed bands that were shared (fig. 2); two bands of c. 55 and c. 65 Kda were present in all strains studied.

Immunoblotting with MAbs 36.4 and 36.3 revealed reactivity with a band of 220–230 Kda (fig. 3a and b), and also with a 110-Kda band. The latter reactivity disappeared when PMSF was used during preparation of the cell-wall polypeptides. Pre-incubation of nitrocellulose paper with rabbit IgG did not influence binding of MAbs 36.3 and 36.4 with the 110-Kda band, indicating a specific binding at this site. Furthermore, the immunoblots confirmed the exclu-

Table. Reactivity of MAbs with clinical isolates of *S. epidermidis* and other bacterial species, as determined in ELISA

MAb no.	Class	Number of strains reactive with MAb			
		<i>S. epidermidis</i> (n = 17 or 44)*	<i>S. hominis</i> (n = 3)	<i>S. aureus</i> (n = 6)	enterobacteria (n = 10)
17.5	IgG ₃	13/17	3	6	0
23.12	IgG ₁	13/17	3	5	0
23.42	IgG _{2a}	10/17	0	5	0
36.1	IgG _{2b}	12/17	1	2	0
36.3	IgG ₁	28/44	2	0	0
36.4	IgG ₁	1/44	0	0	0
17.15	IgM	17/17	3	6	9
23.23	IgM	17/17	2	6	0
36.2	IgM	16/17	1	6	9

Fig. 1. SDS-PAGE of six *S. epidermidis* strains (lanes 1–6: strains 354, 291, ATCC 35984, 463, 348 and 412), two *S. hominis* strains (lanes 7 and 8: strains 268 and ATCC 35982) and three *S. aureus* strains (lanes 9–11).

sive reactivity of MAb 36.4 with the strain used for immunisation (*S. epidermidis* strain 354), whereas MAb 36.3 also reacted strongly with protein bands of c. 220–230 Kda of two other *S. epidermidis* strains (291 and ATCC 35984) and a band of 160–180 Kda of *S. epidermidis* strain 463. MAb 36.3 also reacted with the *S. hominis* test strain in ELISA, but not in immunoblotting. Strains that did not react with either MAb in

ELISA, also failed to show any reaction with these MAbs in immunoblots.

Phagocytosis and chemiluminescence

Opsonisation of *S. epidermidis* strain 354 with MAb 36.4 showed significantly greater phagocytosis ($p < 0.01$) by PMNLs (19% SD 6.5%), than when the

Fig. 2. Immunoblots of nine CNS strains after incubation with rabbit hyperimmune serum. Lanes 1–7: *S. epidermidis* strains ATCC 35984, 463, 291, 412, 501, 348 and 354; lanes 8 and 9: *S. hominis* strains ATCC 35982 and 268.

Fig. 3. Immunoblots of nine CNS strains: (a) after incubation with MAb 36.4, which reacted only with *S. epidermidis* strain 354 (lane 1); (b) after incubation with MAb 36.3 showing reactivity with a high mol. wt band of *S. epidermidis* strain 354 (lanes 1 and 1a); lanes 2-9, *S. epidermidis* strains 291, ATCC 35984, 463, 348, 412 and 501.

bacteria were opsonised with HPS 1% (13.8% SD 1.2%) or were unopsonised (4.7% SD 2.3%) (fig. 4). Complement-mediated opsonophagocytosis was markedly enhanced in the presence of MAb 36.4 (49% SD 7.7%) than with complement alone (6.1% SD 2.0%).

The same experiments performed with human monocytes showed enhanced phagocytosis of *S.*

epidermidis strain 354 opsonised with MAb 36.4 both with (60% SD 9.7%) and without (55% SD 7.3%) complement. MAb 36.3 and all other MABs tested failed to promote uptake of *S. epidermidis* strain 354 (fig. 4).

Opsonisation of *S. epidermidis* strain 354 with MAB 36.4 resulted in an increased generation of CL by human PMNLs (210 SD 120×10^4 cpm) (fig. 5). Incubation of *S. epidermidis* opsonised with MAB 36.4

Fig. 4. Phagocytosis of *S. epidermidis* strain 354 (2.5×10^8 cfu/ml) by human PMNL ($\square$) (5×10^6 ml) or human monocytes (hatched) after opsonisation with MAB 36.4 alone and with complement (36.4 + C). Controls were *S. epidermidis* strains unopsonised and after opsonisation with human pooled serum (HPS 1%) and complement (C). Bar represents SD.

Fig. 5. Luminol-enhanced CL of human PMNLs after incubation with *S. epidermidis* strain 354 opsonised with MAB 36.4 alone (M) and with complement (M + A). Controls were unopsonised strains (C) and strains opsonised with 5% human pooled serum (P) or complement (A). Bar represents SD.

and complement with PMNL displayed increased CL ($710 \text{ SD } 279 \times 10^4 \text{ cpm}$), which was greater than that generated by HPS 5% ($382 \text{ SD } 219 \times 10^4 \text{ cpm}$). Bacteria opsonised by complement alone generated less CL ($57 \text{ SD } 26 \times 10^4 \text{ cpm}$). In contrast, *S. epidermidis* strain 354 opsonised with MAb 36.3 or other MABs described in the table failed to induce a metabolic burst.

Immuno-gold electronmicroscopy

Immunolabelling of *S. epidermidis* strains with colloidal gold particles complexed with *S. aureus* protein A showed homogeneous binding of MAb 36.4 to the surface of the cell (fig. 6). Some binding of MAb 36.4 to extracellular material was also demonstrated. Immuno-electronmicroscopy with MAb 36.3 also showed homogeneous localisation of the MAb to surface structures and extracellular material.

Discussion

We have characterised two MABs that were specific for *S. epidermidis* strains. Both belonged to the IgG₁ subclass. MAb 36.3 reacted strongly with 28 out of 44 *S. epidermidis* strains, without showing cross-reactivity with other gram-positive species, and did not react with gram-negative species. MAb 36.4 reacted only with the strain used for immunisation.

Fig. 6. Immuno-gold electronmicroscopy of *S. epidermidis* strain 354 incubated with MAb 36.4 and labelled with SWAM and protein A-gold. Bar 100 nm.

SDS-PAGE and immunoblotting techniques have been used by some workers to evaluate the usefulness of the technique for typing staphylococci by determining the pattern of cell-wall proteins, and in epidemiological studies of colonisation and infection with *S. epidermidis* strains.^{19,20} In the present study these techniques were used to characterise MABs raised against live staphylococci. We confirmed the results of previous studies²⁰ that the preparation of staphylococcal cell-wall proteins for SDS-PAGE and immunoblotting by lysostaphin digestion of bacterial cells is a reproducible method. Immunoblot analysis revealed reactivity of MABs 36.3 and 36.4 with a band of 220–230 Kda, apparently located on the surface of the strains as indicated by immuno-gold EM studies. Both MABs also showed weak reactivity with a band of c. 110 Kda. Non-specific Fc-binding was ruled out by pre-incubation with normal mouse IgG, which did not interfere with MAB reactivity. Treatment with PMSF abolished reactivity with the 110-Kda band, suggesting that this band is the result of proteolytic degradation.

Reactivity of two different MABs for the same proteins is probably due to the presence of two different epitopes on the same protein complex. In these studies, one of them was specific for *S. epidermidis* strain 354 in ELISA and immunoblot. The other epitope binding MAB 36.3 appeared to be shared by other *S. epidermidis* strains (291, 463 and ATCC 35984). Furthermore, MAB 36.3 appeared to detect mol.-wt variants, because reactivity with *S. epidermidis* strains 463 was with a 160–180-Kda band. Reactivity of one MAB with bands of different mol. wt could be explained either by size heterogeneity of the same protein because of lysostaphin treatment, or to proteolytic digestion of a common protein, or to the presence of variable amounts of associated molecules such as oligosaccharides or fragments of peptidoglycan, in the various strains.

With the phagocytosis assay, we found increased uptake of *S. epidermidis* strain 354 by human PMNLs and monocytes after opsonisation with MAB 36.4, whereas opsonisation with other MABs reactive with CNS did not promote uptake. Opsonisation by complement and MAB 36.4 enhanced bacterial uptake by PMNLs. Complement alone did not promote phagocytosis of *S. epidermidis* strain 354. It is likely that uptake of *S. epidermidis* opsonised with murine IgG₁ MAB 36.4 is mediated by the FcγR2 receptors of human PMNL, whereas uptake by human monocytes is probably mediated by FcγR2 as well as FcγR1.¹ The presence on monocytes of both FcγR1 and FcγR2, which show respectively low and high affinity for murine IgG₁ immunoglobulins, might explain the higher uptake of *S. epidermidis* strain 354 opsonised with MAB 36.4 in the absence of complement by monocytes compared to PMNLs. Opsonisation of *S. epidermidis* was enhanced in the presence of complement, but complement alone did not promote uptake by PMNLs as well as by monocytes. Clark and

Easmon²¹ also found that complement did not promote interaction of CNS with human PMN. In contrast, Van Bronswijk *et al.*⁸ found that phagocytosis of CNS by human PMNL was promoted by complement alone, although this required extension of the opsonisation time to 90 min.

Opsonisation of *S. epidermidis* strain 354 with MAb 36.4 also stimulated PNML CL. Addition of complement markedly enhanced the CL, whereas opsonisation by complement alone did not result in metabolic activation of PMNL. Together these results indicate that MAb 36.4, when bound to the staphylococcal surface, is capable of binding the phagocyte Fc-receptor and of activating complement.

Immuno-gold electronmicroscopy showed a homogeneous distribution of binding sites for MAbs 36.4

and 36.3 both on and close to the surface of *S. epidermidis* strain 354.

In conclusion, our experiments have demonstrated a MAb (36.4) that reacts with a 220-Kda band in immunoblots and shows complement-independent opsonic activity in phagocytosis studies with PMNLs and monocytes. We postulate that the presence of Fc γ R1 and Fc γ R2 receptors on human monocytes is responsible for the higher uptake by these phagocytes. Furthermore, we have shown that MAb 36.4 is capable of generating the metabolic burst in human PMNLs by a complement independent mechanism. Future experiments will be directed at identification and biochemical characterisation of the antigen which appears to be important in the opsonic requirements of CNS.

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