IMMUNOLOGICAL RESPONSE TO INFECTION

Antibody response to *Staphylococcus aureus* collagen binding protein in patients with *S. aureus* septicaemia and collagen binding properties of corresponding strains

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Summary. An ELISA was developed for the detection of IgG antibodies to *Staphylococcus aureus* collagen binding protein (CnBP) in 95 patients with *S. aureus* endocarditis, complicated septicaemia with bone and joint involvement or uncomplicated septicaemia and in 95 control patients. Sixty percent of *S. aureus*-infected patients showed a positive peak anti-CnBP antibody level or a significant rise in titre, or both, during infection, but patients with *S. aureus* endocarditis or complicated septicaemia could not be differentiated from those with uncomplicated *S. aureus* septicaemia. The collagen binding capacity of *S. aureus* strains from 82 of the 95 patients was investigated in a particle agglutination assay and a ^{125}I -labelled assay. All strains bound collagen in the particle agglutination assay as did 68 % in the ^{125}I -labelled assay, but there was no correlation between collagen binding of the patient strain and endocarditis, joint or skeletal involvement. An anti-CnBP antibody response was seen in 16 patients infected with a *S. aureus* strain negative for collagen binding *in vitro*, indicating in-vivo expression of CnBP.

Introduction

Specific attachment by various pathogens to extracellular matrix (ECM) proteins are important steps in early tissue colonisation.¹ *Staphylococcus aureus* binds to several ECM proteins such as fibronectin,² fibrinogen,³ heparan sulphate,⁴ vitronectin,⁵ thrombospondin⁶ and various collagens.^{7,8} *S. aureus* is a common pathogen in bone and joint infection as well as in infective endocarditis,⁹ and collagen type I is a major component of bone, cartilage and endocardium.¹⁰ Recent animal studies have suggested that the collagen binding properties of *S. aureus* strains are correlated with the development of septic arthritis.¹¹ A 135-kDa collagen binding protein (CnBP) from *S. aureus* Cowan 1 has been purified, characterised and shown to be highly immunogenic in rabbits,¹² and the gene for this protein has been sequenced and cloned.¹³ In the present studies an ELISA was developed with CnBP as antigen to determine the diagnostic value of IgG antibodies to CnBP in patients with *S. aureus* septicaemia with and without endocarditis or bone and joint involvement and in control patients. The collagen binding capacity of *S. aureus* strains isolated

from patients with endocarditis, complicated septicaemia with bone and joint involvement, and uncomplicated septicaemia was determined.

Materials and methods

Chemicals

Tween-20 was obtained from Kebo AB, Spånga, Sweden. Collagen binding protein (CnBP) was purified as described previously¹² by digestion of bacteria with lysostaphin, ion-exchange chromatography, ammonium sulphate precipitation and gel filtration. It was a kind gift from Dr L. Switalski, School of Dental Medicine, University of Pittsburgh, PA, USA. Rabbit anti-human IgG labelled with alkaline phosphatase was purchased from Dakopatt, Glostrup, Denmark and *p*-nitrophenyl phosphate from Sigma; bovine collagen (Vitrogen 100^R containing collagen I 95 % and collagen III 5 %) was purchased from Collagen Corporation, Palo Alto, CA, USA; latex beads were purchased from Difco; ^{125}I iodine was purchased from Amersham Corp.; iodobeads were obtained from Pierce Chemical Co., Rockford, IL, USA and bovine serum albumin (BSA) from Boehringer GmbH, Mannheim, Germany.

Table I. Age and sex distribution in the various patient groups

Diagnosis (number of strains)	Mean age (range) (years)	Male/female
<i>S. aureus</i> endocarditis (42)	60 (22–87)	25/17
Complicated <i>S. aureus</i> septicaemia (26)	63 (20–86)	19/7
Uncomplicated <i>S. aureus</i> septicaemia (27)	64 (24–91)	17/10
Non- <i>S. aureus</i> endocarditis (9)	62 (25–86)	1/8
Non- <i>S. aureus</i> septicaemia (31)	67 (21–89)	10/21
Febrile controls (55)	54 (17–91)	26/29

Patients

All patients were febrile with a temperature $> 38.5^{\circ}\text{C}$ for at least 2 days. In all patients with endocarditis or septicaemia, or both, at least two blood cultures were positive.

S. aureus endocarditis. Forty-two patients had definite, probable or possible endocarditis according to the criteria of von Reyn *et al.*¹⁴ Those who underwent cardiac surgery were regarded as having definite endocarditis if macroscopic vegetations were present on the valves. Three patients had definite, 18 probable and 21 possible endocarditis.

S. aureus complicated septicaemia. Twenty-six patients developed infectious involvement of bone or joints as verified by needle aspiration with positive culture, scintigram or X-ray.

S. aureus uncomplicated septicaemia. Twenty-seven patients lacked all signs of bone and joint infection or endocarditis. One patient had septicaemia derived from an intravenous catheter.

Non-*S. aureus* endocarditis. Nine patients had endocarditis caused by bacteria other than *S. aureus*. Two of these were definite, caused by *Enterococcus faecalis* and coagulase-negative staphylococci, one had probable endocarditis caused by *Streptococcus sanguis* and six had possible endocarditis caused by *Str. sanguis* ($n = 2$), *Str. mitis* (1), *Str. milleri* (1), β -haemolytic streptococci group G (1) and *Str. pneumoniae* (1), respectively.

Non-*S. aureus* septicaemia. Thirty-one patients had septicaemia due to *Escherichia coli* ($n = 8$), *Str. pneumoniae* (5), β -haemolytic streptococci (5), *Pseudomonas aeruginosa* (4), coagulase-negative staphylococci (4), *Ent. faecalis* (2), *Salmonella* spp. (2) and *Proteus mirabilis* (1).

Febrile controls. Fifty-five patients with temperatures $> 38.5^{\circ}\text{C}$ for at least 2 days and two or more negative blood cultures showed no clinical signs or bacteriological evidence of *S. aureus* infection. Diagnoses were pneumonia ($n = 27$), urinary tract infection (11), erysipelas (7), gastro-enteritis (3), viral infection (3), upper respiratory tract infection (2), sigmoiditis (1) and fever during leukopenia caused by treatment with cytostatic drugs (1). For age distribution and male: female ratio in the various patient groups, see table I.

Serum samples

Sera were collected at the Departments of Infectious Diseases, University Hospital of Lund and Örebro Medical Centre Hospital, and stored in divided volumes at -20°C . Acute sera were drawn within 10 days and convalescent sera 11–30 days after onset of infection (usually onset of fever).

Two hundred and twenty-nine sera were analysed from 95 patients with *S. aureus* septicaemia; in 17 patients only convalescent sera were available. Eighty serum samples were analysed from 40 patients with non-*S. aureus* endocarditis or septicaemia and from three patients only convalescent sera were available. In the febrile non-septicaemic control group paired sera were available from all 55 patients.

CnBP-ELISA

Purified CnBP was dissolved in phosphate-buffered saline (PBS, pH 7.2) to a concentration of $0.5\text{ }\mu\text{g/ml}$. Wells of microtitration plates (Maxisorb Nunc, Roskilde, Denmark) were coated with $100\text{ }\mu\text{l}$ of the antigen solution. Plates were incubated at 37°C for 1 h and washed. Patients' sera were diluted 1 in 2000 in PBS with Tween-20 0.05%, and $100\text{ }\mu\text{l}$ were incubated in each well for 1 h at 37°C . After another washing, $100\text{ }\mu\text{l}$ of rabbit anti-human IgG diluted 1 in 2000 in PBS-Tween 0.05% were added to each well. Plates were incubated for 1 h on a shaker at room temperature. Finally, plates were washed, incubated with *p*-nitrophenyl phosphate for 30 min and then read at 405 nm on a spectrophotometer (Multiskan Plus, Labsystems, Helsinki, Finland). Results were related to a positive control and expressed as the ELISA index.

Bacterial strains

S. aureus septicaemia strains were grown in blood culture bottles, isolated on blood agar plates and stored in calf serum at -20°C until use. Seventeen *S. aureus* strains were available from 18 patients with probable endocarditis, 19 of 21 strains from patients with possible *S. aureus* endocarditis, 23 of 26 strains from patients with complicated and 23 of 27 strains from patients with uncomplicated *S. aureus* septicaemia, respectively. No strains were available from patients with definite endocarditis.

Collagen binding assays

Particle agglutination assay. A previously described particle agglutination assay (PAA)¹⁵ was used to screen collagen binding properties of 82 *S. aureus* strains. The strains were cultured on blood agar which has been shown to increase collagen binding capacity as compared to other media.¹⁵ *S. aureus* strain Cowan 1 served as a positive control. Strains were suspended in 0.02 M potassium phosphate buffer (pH 6.8) to

c. 10^{10} cells/ml. Latex beads were coated with bovine collagen. One drop of bacterial suspension was mixed with coated latex beads in 0.17 M glycine NaOH buffer (pH 6.8) on a glass slide. Strains were tested for auto-aggregation by mixing them with uncoated latex beads. Results were scored from 3+ when strains agglutinated within 30 s, 2+ within 1 min, 1+ within 2 min to negative (—) when there was no agglutination within 2 min.¹⁶

¹²⁵I-collagen binding assay. Bovine collagen was labelled with ¹²⁵I according to a modified Chloramine-T method with Iodobeads^R.¹⁶ *S. aureus* strains were cultured for 24 h at 37°C on blood agar, harvested, washed and resuspended in PBS to c. 5×10^9 cfu/ml. ¹²⁵I-collagen was diluted to c. 0.3×10^4 cpm/50 µl in PBS with BSA 1% and added to 100 µl of bacterial suspension. After mixing, tubes were incubated for 1 h at room temperature. The binding reaction was stopped by adding 2 ml of PBS + Tween-20 0.1% v/v. After centrifugation at 3000 rpm for 15 min the supernate was aspirated and the radioactivity of the pellet was measured in a 1260 Multigamma counter (LKB-Wallac, Turku, Finland). Results were expressed as percentage radioactivity of total amount of ¹²⁵I-collagen added. *S. aureus* Cowan 1 served as a positive control.

Statistical analysis

For evaluation of the reproducibility of an assay, the analytical error was calculated. The inter-assay error was expressed as the standard deviation of the single determination $s = \pm \sqrt{(Sd^2/2n)}$ where Sd^2 equals the squared sum of differences within pairs and n equals the number of pairs. Since the absolute differences within paired observations in the CnBP-ELISA rose with higher optical density values, the standard deviations of the single determinations in this assay were calculated as percentage values. The χ^2 test was used to compare differences in anti-CnBP levels in various patient groups and collagen binding capacity in strains from patients with *S. aureus* endocarditis, complicated and uncomplicated septicaemia.

Results

The upper normal anti-CnBP IgG level was defined as the mean value of the febrile control group + 2 SD. This gave an upper normal ELISA index of 0.712. As shown in fig. 1 and table II, peak anti-CnBP IgG levels above cut-off were found in 12 (57%) of 21 patients with definite or probable *S. aureus* endocarditis, 9 (43%) of 21 patients with possible *S. aureus* endocarditis, 9 (35%) of 26 patients with *S. aureus* complicated septicaemia, 8 (30%) of 27 patients with *S. aureus* uncomplicated septicaemia and 3 (5%) of 55 febrile control patients. No patients with non-*S. aureus* endocarditis or septicaemia showed a positive peak

antibody level. The analytical error of the method was 21% and a significant rise in titre between acute and convalescent sera was defined as > 50% increase of ELISA index. Positive peak anti-CnBP titres or a significant titre rise, or both, were found in 43 (63%) of 68 *S. aureus* infected patients with endocarditis or bone and joint infection as compared to 14 (52%) of 27 patients with uncomplicated *S. aureus* septicaemia (table II). This difference was not significant ($p > 0.05$). No patients with non-*S. aureus* endocarditis was positive, but two patients with *P. aeruginosa* and one with *Str. pneumoniae* septicaemia showed significant rises in titre. Thus, the specificity for diagnosing *S. aureus* endocarditis or septicaemia, or both, was 92% (37 of 40) in the present studies.

Six *S. aureus* strains auto-aggregated in the PAA and all the remaining 76 strains bound collagen (table III). Sixty-two strains were run on several different occasions, and 13 (21%) strains changed from 1+ to 2+ or 3+ or *vice versa*. The analytical error of the ¹²⁵I-collagen binding assay was 2.5%, background was 1.7% and values below $2 \times 2.5\% + 1.7\% = 6.7\%$ were regarded as negative for collagen binding. Fourteen (82%) of 17 *S. aureus* strains from patients with probable endocarditis were positive as were 12 (63%) of 19 strains from patients with possible endocarditis, 16 (70%) of 23 strains from patients with complicated and 15 (65%) of 23 strains from patients with uncomplicated *S. aureus* septicaemia (table III). There was poor correlation between the two collagen binding assays where 10 of 37 strains classified as 3+ showed no ¹²⁵I-collagen binding capacity (fig. 2).

There was no significant correlation ($p > 0.05$) between the development of endocarditis or joint and skeletal infection and the collagen binding capacity of corresponding strains of *S. aureus* with either collagen binding assay (table III). Fifty-one strains were available from patients seropositive for anti-CnBP, among which 16 (31%) were found negative in the ¹²⁵I-collagen binding assay. The patients infected with these 16 strains developed positive peak anti-CnBP antibody level ($n = 8$), rise in titre (3) or both (5).

Discussion

Many serological assays with various *S. aureus* antigens have been published over the last decade with the purpose of finding an assay or a combination of assays that could distinguish patients with complicated septicaemia, including endocarditis and deep-seated tissue infections, from those with uncomplicated septicaemia.¹⁷⁻²¹ No single assay has so far proved to be adequate for this differential diagnosis, but combinations of assays have in some cases reached acceptable predictive values.²²⁻²⁵

In the present studies an ELISA was developed to determine the IgG antibody response towards *S. aureus* CnBP. Detectable antibodies to CnBP were

Fig. 1. Levels of IgG antibody to *S. aureus* collagen binding protein (CnBP) measured by ELISA. Peak antibody levels are shown from patients with *S. aureus* endocarditis (A, $n = 42$), *S. aureus* complicated septicaemia (B, $n = 26$), *S. aureus* uncomplicated septicaemia (C, $n = 27$), non-*S. aureus* endocarditis (D, $n = 9$), non-*S. aureus* septicaemia (E, $n = 31$) and febrile controls (F, $n = 55$); ----, upper normal anti-CnBP-IgG level defined as mean value of the febrile control group $+ 2$ SD.

Table II. Number of patients with positive peak anti-CnBP ELISA index or significant rise in titre during the course of infection, or both

Diagnosis	Number of patients	Number (%) of patients with	
		Positive peak ELISA index	Positive peak ELISA index or significant titre rise, or both
<i>S. aureus</i> definite or probable endocarditis	21	12 (57)	15 (71)
<i>S. aureus</i> possible endocarditis	21	9 (43)	15 (71)
<i>S. aureus</i> complicated septicaemia	26	9 (35)	13 (50)
<i>S. aureus</i> uncomplicated septicaemia	27	8 (30)	14 (52)
Non- <i>S. aureus</i> endocarditis	9	0 (0)	0 (0)
Non- <i>S. aureus</i> septicaemia	31	0 (0)	3 (10)
Febrile controls	55	3 (5)	3 (5)

found in all *S. aureus*-infected patients as well as in controls (fig. 1), which has also been reported with serological assays with various *S. aureus* antigens.¹⁷⁻²¹ Cross-reacting antibodies in non-*S. aureus* endocarditis or septicaemia were seen in only 3 (8%) of 40 patients, two patients with *P. aeruginosa* septicaemia and one patient with *Str. pneumoniae* septicaemia showing a significant rise in titre although not reaching a positive peak antibody level. Thus, this assay seems to be highly specific for *S. aureus* infection, and

contrary to, for example, assays with *S. aureus* teichoic acid, peptidoglycan, crude *S. aureus* antigen, whole *S. aureus* cells and α -toxin,^{20-22, 24} no cross-reacting antibodies were seen in patients with endocarditis or septicaemia due to α -streptococci, enterococci or coagulase-negative staphylococci. This could be due to the use of a highly purified protein specific for *S. aureus*.

The overall sensitivity in diagnosing *S. aureus* endocarditis or septicaemia was 60% (57 of 95) (table

Table III. Binding of collagen to *S. aureus* strains from patients with endocarditis, complicated and uncomplicated septicaemia

Diagnosis	Number of strains	Number (%) of particle agglutinating strains			Number (%) of ¹²⁵ I-collagen binding strains
		+++	++	+	
Probable endocarditis	17	7 (41)	2 (12)	6 (35)	14 (82)
Possible endocarditis	19	8 (42)	5 (26)	5 (26)	12 (63)
Complicated septicaemia	23	15 (65)	2 (9)	5 (22)	16 (70)
Uncomplicated septicaemia	23	7 (30)	7 (30)	7 (30)	15 (65)

Six strains autoagglutinated in the PAA, two were from patients with probable endocarditis, one from a patient with possible endocarditis, one from a patient with complicated septicaemia and two from patients with uncomplicated septicaemia.

Fig. 2. Correlation between collagen binding measured with ¹²⁵I-collagen and particle agglutination assay in *S. aureus* strains from patients with endocarditis, complicated and uncomplicated septicaemia. Values below the dashed line (---) indicate negative result in the ¹²⁵I-collagen binding assay.

II), which is similar to the results of several other serological assays^{17-19, 21} and indicates that CnBP is highly immunogenic. It was assumed that patients with *S. aureus* endocarditis or complicated septicaemia would show increased anti-CnBP levels based on the expected increase in CnBP expression when *S. aureus* adheres to the collagen of endocardium, bone and cartilage. However, there was no significant difference in antibody levels among these patients as compared to those with *S. aureus* uncomplicated septicaemia ($p > 0.05$), and thus the anti-CnBP ELISA could not be used as a single assay to differentiate between such patient groups.

In previous studies a correlation between *S. aureus* endocarditis, arthritis and osteomyelitis and high collagen binding capacity of corresponding *S. aureus* strains has been suggested. In an animal model, intravenous challenge of *S. aureus* strain Phillips expressing CnBP caused septic arthritis in all of 10 mice, as compared to 5 of 10 mice challenged with a mutant strain not expressing CnBP.¹¹ With ¹²⁵I-collagen, Holderbaum *et al.*²⁶ have previously reported a significantly higher proportion (26 of 49) of high collagen binding strains in bacteraemic patients with *S. aureus* endocarditis, arthritis and osteomyelitis as compared to those with no evidence of metastatic

infection (13 of 41). The authors did not give diagnostic criteria for their patients with endocarditis, joint or skeletal infection and their septicaemic patients without signs of metastatic infections all had infected intravenous catheters. In the present studies, a similar ^{125}I -collagen binding assay was used but no difference in the collagen binding capacity of *S. aureus* strains from patients with or without endocarditis or bone or joint infection was found (table III). One possible reason for this discrepancy could be that *S. aureus* strains colonising intravenous catheters differ in their collagen binding capacity from other septicaemia strains. However, all strains studied, except for six autoagglutinating strains, were positive in the PAA, and it could be argued that the PAA better reflects in-vivo conditions (collagens do not appear in solution *in vivo*¹⁰).

Furthermore, 16 patients infected with *S. aureus* strains negative in the ^{125}I -collagen binding assay who had positive anti-CnBP antibody levels ($n = 8$), significant rises in titre ($n = 3$) or both ($n = 5$) were found. These results indicate in-vivo expression of CnBP in in-vitro negative strains. It is of interest to note that inconsistencies between antibody response and expression of *S. aureus* antigen have been reported previously. Roloff *et al.* found no significant difference in lipase activity between septicaemic strains from 22

patients positive in an anti-lipase ELISA and strains from 16 serologically negative patients.²⁷ In a recently published study no correlation was demonstrated between antibody response to α -toxin in patients with *S. aureus* septicaemia and α -toxin production *in vitro* by the corresponding strain.²⁸ However, in both these studies all strains produced at least low amounts of the antigens.

In conclusion, in patients with *S. aureus* septicaemia we were unable to show a correlation between the bone, joint or cardiac involvement and the in-vitro collagen binding capacity of the patient strain. The anti-CnBP ELISA was highly specific for *S. aureus* infection and might aid in the diagnosis of *S. aureus* endocarditis or septicaemia but cannot be used as a single assay in differentiating between endocarditis or complicated septicaemia and uncomplicated septicaemia. An ongoing study will determine the value of the anti-CnBP ELISA in combination with various other serological assays in the diagnosis of serious *S. aureus* infections.

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