Isolation and Characterization of a Putative Collagen Receptor from *Staphylococcus aureus* Strain Cowan 1*

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In a previous study we demonstrated that cells of *Staphylococcus aureus* strain Cowan bind ^{125}I-collagen in a receptor-ligand type of interaction (Speziale, P., Raucci, G., Visai, L., Switalski, L. M., Timpl, R., and Höök, M. (1986) *J. Bacteriol.* 167, 77–81). In the present communication we report on the isolation and preliminary characterization of a putative collagen receptor from a lysate of *S. aureus* strain Cowan. Antibodies raised against a collagen receptor positive strain inhibit the binding of ^{125}I-collagen to bacterial cells, whereas antibodies raised against a collagen receptor negative strain were without effect. Solubilized cell surface components did not exhibit any measurable affinity for collagen-Sepharose. However, the inhibitory effect of the antibodies against bacterial cells was neutralized by the lysate from a receptor-positive but not receptor-negative strain. A collagen receptor assay was designed based on this observation and used to develop a receptor purification protocol involving anion exchange chromatography, ammonium sulfate precipitation, and gel chromatography. Using this procedure a protein with an apparent M_r of 135,000 was purified. This protein which was present on a collagen receptor-positive strain but not on a receptor-negative strain could completely neutralize the inhibitory activity of the antibodies raised against *S. aureus* strain Cowan. Furthermore, antibodies raised against the 135-kDa protein inhibited the binding of collagen to bacteria, and this protein is tentatively identified as a collagen receptor.

Many potentially pathogenic microorganisms bind to extracellular matrix proteins such as fibrinogen, fibronectin, and laminin. It is believed that these interactions represent mechanisms of host tissue adherence. Bacterial cells possess specific receptor[1] molecules on their surface which bind to distinct sites in the matrix proteins of the host.

A bacterial cell may simultaneously express receptors (adhesins) for several matrix proteins. *Staphylococcus aureus* strains may independently bind fibronectin (Kuusela, 1978; Switalski *et al.*, 1983), fibrinogen (Hawiger *et al.*, 1982), laminin (Lopes *et al.*, 1985), vitronectin (Chhatwal, 1987), and collagen. In some instances these proteins have been shown in *in vitro* assays to serve as substrates for bacterial adherence (Herrmann *et al.*, 1988; Kuusela *et al.*, 1985; Vaudaux *et al.*, 1984; Vercellotti *et al.*, 1984; Wadström *et al.*, 1987). So far the best characterized bacterial receptor for a matrix protein is the *S. aureus* receptor for fibronectin. This receptor isolated from bacterial cells solubilized with lysostaphin is multivalent and migrates as a protein with an apparent M_r of 210,000 when analyzed on PAGE[2] in SDS (Fröman *et al.*, 1987). The gene coding for this has been cloned (Flock *et al.*, 1987) and sequenced (Signäs *et al.*, 1989). The fibronectin binding activity of the receptor has been localized to a domain which is composed of a 38-amino acid unit repeated 3 times. Synthetic peptides mimicking these three sequences interact with fibronectin as indicated by their ability to inhibit binding of fibronectin to intact bacteria (Signäs *et al.*, 1989).

Previous studies on the binding of collagen to *S. aureus* cells have shown that staphylococci may recognize collagens regardless of type and species of origin (Carret *et al.*, 1985; Holderbaum *et al.*, 1986; Mamo *et al.*, 1988; Vercellotti *et al.*, 1985). Moreover, collagen receptor-positive strains of staphylococci also recognize isolated α chains and peptides generated by CNBr cleavage of α chains of type I collagen. Synthetic polypeptides (PGP)$_n$ mimicking the structure of collagen were found to inhibit the binding of collagen to bacteria, suggesting that staphylococci recognize repetitive structures in the collagen molecule (Speziale *et al.*, 1986).

A collagen receptor from bacteria has previously not been identified. In this communication we report on the isolation and characterization of a *S. aureus* surface protein which tentatively is identified as a collagen receptor.

[1] As we have pointed out in our previous paper (Fröman *et al.*, 1987), we use the term "receptor" referring to the components on the bacterial cell surface interacting with collagen. This is contrary to some other reports where this term describes components of the host tissue, which bacterial "adhesins" recognize. Although our receptors do not fulfill all the criteria traditionally associated with the receptors of eukaryotic cells, we feel that referring to extracellular matrix proteins as receptors is improper; they resemble the eukaryotic receptors functionally in their ability to interact with host components. After proving that this interaction plays a role in bacterial attachment to host tissues, bacterial "collagen receptor" can be referred to as a "collagen adhesin."

MATERIALS AND METHODS

Collagen—Collagen type II isolated from chicken sternum (Reese and Mayne, 1981) was a gift from Dr. Richard Mayne, University of Alabama at Birmingham. Na^{125}I, specific activity 15 mCi/μg, was obtained from Amersham Corp. Collagen was labeled with ^{125}I by the chloramine-T method (Hunter, 1978). Although the content of tyrosine in collagen is low, we have observed that extending the labeling time to 3 min yields ^{125}I-collagen of high specific activity, which migrates identically with unlabeled collagen on PAGE in SDS. The specific activity of the labeled protein was typically 0.5–1.0 × 10⁶ cpm/μg.

Antibodies—Antibodies against whole cells of *S. aureus* strains Cowan and Newman were raised in New Zealand rabbits employing

[2] The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

the immunization protocol of Löfkvist and Sjöquist (1963). Polyclonal antibodies against purified collagen receptor were also raised in rabbits by three intramuscular injections of 50 µg of receptor protein emulsified in Freund's incomplete adjuvant (Difco) at 10-day intervals. Rabbits were bled at 2-week intervals. The IgG fraction of serum was purified by affinity chromatography on protein A-Sepharose, eluted with 3 M MgCl$_2$, dialyzed to PBS (0.14 M NaCl, 10 mM phosphate, 0.02% azide, pH 7.4), and stored in small aliquots.

Fab fragments were generated by digestion of IgG with papain coupled to Sepharose (PBS buffer, 5 mg of papain/100 mg of IgG), at 37 °C overnight, and Fc fragments were separated on protein A-Sepharose. In some experiments Fab fragments were absorbed with *S. aureus* cells. Five hundred µg of Fab fragments were absorbed with 50 mg (wet weight) of heat-killed bacteria for 1 h at 37 °C and overnight at 4 °C. The incubation mixtures were centrifuged, and the clear supernatant was collected and stored at −20 °C.

Bacteria—*S. aureus* strains Cowan and Newman were cultured under constant rotation for 15 h at 37 °C in brain heart infusion broth (Difco). Bacteria were harvested by centrifugation, suspended in PBS, and heated at 88 °C for 20 min to kill bacteria and inactivate hydrolases. Bacteria were collected by centrifugation and resuspended in PBS to a final density of 1 × 10^{10} cells/ml. The number of cells was determined by comparing the absorbance of the sample with a previously prepared standard curve relating A_{600} to the cell number determined by counting cells in a Petroff Hausser chamber.

^{125}I-Collagen Binding Assay—The amount of ^{125}I-collagen bound to bacteria was quantified essentially as described previously (Fröman *et al.*, 1987), except that the number of bacterial cells/incubation mixture was reduced. Briefly, 10^8 cells of *S. aureus* strain Cowan in a total volume of 0.5 ml containing 5 × 10^4 cpm of ^{125}I-collagen type II, 0.1% bovine serum albumin, and 0.1% Tween 80 in PBS were incubated for 1 h at 20 °C. The reaction was stopped by the addition of 3 ml of ice-cold PBS containing 0.1% Tween 80, and the tubes were immediately centrifuged at 1350 × g for 20 min. After aspiration of the supernatant the pellet remaining in the tubes was analyzed for radioactivity in a γ counter. Duplicate samples were analyzed, and background values representing radioactivity recovered in the tubes incubated in the absence of bacteria were subtracted.

Assay of Collagen Receptor—In initial experiments lysostaphin-solubilized bacterial cell surface components were found not to inhibit the binding of ^{125}I-collagen to *S. aureus* cells. Solubilized receptors could therefore not be detected or quantified in a conventional inhibition assay, and an alternative method had to be developed. Antibodies against collagen receptor-positive cells of *S. aureus* strain Cowan were found to inhibit the binding of ^{125}I-collagen to bacteria. If we assume that the effect of the antibodies is the consequence of an antibody binding to collagen receptors, we can measure molecules containing the active epitope (*e.g.* receptors) in an assay where soluble cell surface components are used to neutralize the inhibitory activity of the antibodies. Similar assays have previously been used to quantify adhesion molecules on *Dictyostelium discoideum* (Müller and Gerisch, 1978) and vertebrate cells (Ocklind and Öbrink, 1982; Urushihara and Takeichi, 1980). To make this assay as sensitive as possible, the amount of anti-*S. aureus* Cowan Fab fragments causing 80% inhibition of ^{125}I-collagen binding to intact bacterial cells in a standard assay described above was determined and used as a base line. Depending on the batch of antibodies (preabsorbed with cells of strain Newman) this amount ranged from 9 to 17 µg of Fab. Antibodies were preincubated with varying amounts of solubilized receptor preparations for 30 min at 37 °C. The mixture was subsequently added to intact bacteria and incubated with ^{125}I-collagen as described above. The degree of neutralization of inhibitory activity of antibodies plotted *versus* the log of added lysate typically gave a linear graph. The amount of material reversing the inhibitory activity of antibodies by half (*i.e.* from 80% inhibition to 40% inhibition) was arbitrarily defined as one receptor unit.

Solubilization of Bacterial Cell Surface Component—Bacterial components were solubilized with lysostaphin in the presence of protease inhibitors essentially as described previously for the isolation of fibronectin receptor (Fröman *et al.*, 1987) except that bacterial cells were sonicated before treatment with enzymes. Briefly, 10 g (wet weight) of bacteria grown as described above were suspended in 100 ml of TBS (0.14 M sodium chloride, 50 mM Tris-HCl, pH 7.4) and sonicated for 3 min (Heat Systems Ultrasonics, Plainview, NY). The suspension was supplemented with 1 mg of lysostaphin, 2 mg of DNase, 1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide and incubated under constant rotation at 37 °C. After 30 min a second 1 mg of lysostaphin was added, and the incubation was

continued for an additional 2.5 h. The enzyme digestion was stopped by heating the incubation mixture for 10 min at 88 °C. Bacterial debris was removed by centrifugation (20 min, 5000 × g), and the pH of the supernatant (referred to in the text as the "lysate") was adjusted to 7.4. The supernatant was stored at −20 °C until used.

Electrophoresis and Western Blotting—Electrophoresis in polyacrylamide gel was performed according to Blobel and Dobberstein (1975). The samples were boiled in a buffer containing 4% sodium dodecyl sulfate, 2 mM dithiothreitol, 30% sucrose, 0.01% bromphenol blue, 82 mM Tris-HCl followed by alkylation with iodoacetamide. The gels were stained with Coomassie Brilliant Blue R-250 and dried.

Blotting followed basically the procedure of Towbin *et al.* (1979). Proteins separated by electrophoresis were electroblotted for 2.5 h at 400 mA onto Immobilon-P membranes (Millipore, Bedford, MA). Additional binding sites were blocked by incubating the membrane with 1% defatted bovine milk in TBS for 2 h at 22 °C. The membranes were incubated with purified Fab fragments (100–200 µg/100 ml) in TBS, containing 0.1% milk for 2 h at 37 °C, and washed with TBS. Subsequently membranes were incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-Rad) diluted 1:3000 with TBS containing 0.1% milk. Enzyme-conjugated antibody was detected by a color reaction using 4-chloro-1-naphthol as a substrate according to the manufacturer's instructions.

RESULTS

Binding of ^{125}I-Collagen to Enzyme-digested S. aureus Cells

Staphylococcal cells were digested with different enzymes in attempts to release the collagen receptor. Residual binding by enzyme-treated cells was quantified and compared with that of untreated bacteria (Fig. 1). Bacterial cells digested with proteolytic enzymes, trypsin, thermolysin, and papain, lost essentially all collagen binding activity as did bacteria treated with peptidoglycan degrading enzyme, lysostaphin. On the other hand digestion with lysozyme cleaving only the carbohydrate chain of the peptidoglycan did only marginally reduce the ^{125}I-collagen binding activity of the treated bacteria.

Material released from bacteria during enzyme digestion was tested for its ability to inhibit ^{125}I-collagen binding to intact cells. Somewhat surprisingly none of the preparations tested including the lysate generated by lysostaphin digestion (shown in the past (Fröman *et al.*, 1987) to contain *e.g.* active fibronectin receptors or protein A) significantly interfered with the binding of collagen to bacteria (data not shown). Since these digests should contain solubilized receptors or their fragments, a direct inhibition assay to quantify collagen receptors in these digests is not possible. Alternative receptor assays therefore had to be explored.
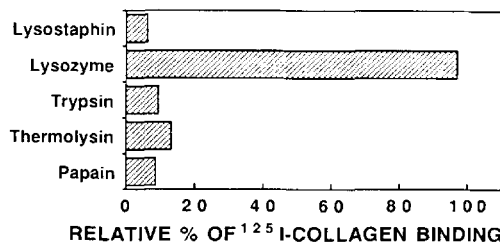

FIG. 1. **Solubilization of ^{125}I-collagen binding components from *S. aureus* Cowan cells.** Bacteria, 10^{10} cells in 1 ml of PBS, were incubated with 20 µg of each enzyme for 2 h at 37 °C. The incubation mixture with papain was supplemented with 2 mM EDTA and 10 mM cysteine. The digestion was stopped by boiling the samples for 10 min at 37 °C, and the mixtures were centrifuged. Bacterial pellets suspended in the initial volume of PBS were assayed for their ability to bind ^{125}I-collagen in the standard assay. Data are expressed relative to control, *i.e.* bacteria incubated in the absence of enzymes.

Inhibition of ^{125}I-Collagen Binding to S. aureus Cells by Antibodies

Antibodies against whole cells of collagen receptor-positive strain Cowan and collagen receptor-negative strain Newman were raised in rabbits. IgG and Fab fractions of these antibodies were tested as potential inhibitors of ^{125}I-collagen binding to *S. aureus* Cowan cells (Fig. 2). Only immune anti-Cowan antibodies effectively inhibited binding of ^{125}I-collagen to bacteria. The amount of collagen bound was reduced by half when 10^8 bacterial cells were incubated with 15 μg of anti-Cowan Fab fragments, and 50 μg of the same antibodies caused 90% inhibition of binding. No significant inhibition of ^{125}I-collagen binding was obtained by preimmune antibodies or by antibodies raised against cells of strain Newman. This observation suggested that anti-Cowan antibodies, which block ^{125}I-collagen binding, recognize epitopes on the collagen receptor located at or close to the collagen binding site. Inhibition of collagen binding was observed with both intact IgG (not shown) and Fab fragments. To avoid any possible interference of protein A with the antibodies, we routinely used Fab fragments rather than IgG in our assays. Antibodies against other previously identified staphylococcal cell wall components, *e.g.* fibronectin receptor or protein A, had no effect (data not shown).

Anti-Cowan and anti-Newman Fab fragments were absorbed with cells of strain Cowan or Newman. The remaining antibodies were tested for their ability to inhibit the binding of ^{125}I-collagen to cells of strain Cowan (Fig. 3). The inhibitory activity of anti-Cowan antibodies could be neutralized by absorption with homologous bacteria, while absorption with cells of a collagen receptor-negative strain (Newman) only marginally reduced the inhibitory activity of the antibody preparation. Hence, the epitope(s) which the inhibiting antibodies recognize is present on a collagen receptor-positive strain but not on a receptor-negative strain.

A Collagen Receptor Assay

If the ^{125}I-collagen binding inhibitory activity of anti-Cowan antibodies may be specifically absorbed using whole cells of collagen receptor-positive bacteria, it may also be possible to absorb this activity using solubilized bacterial components. To test this hypothesis anti-Cowan antibodies were incubated with bacterial components solubilized by lysostaphin digestion of bacterial cells of strains Cowan and Newman, respectively, and then subsequently assayed for their ability to
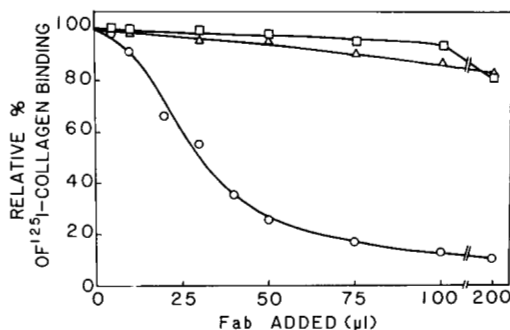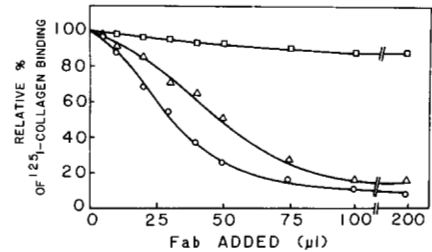
FIG. 3. **Absorption of inhibitory activity of polyclonal anti-*S. aureus* and anti-collagen receptor antibodies with homologous and heterologous bacteria.** Fab fragments (500 μg/2.5 ml) against *S. aureus* Cowan were preincubated with 50 mg (wet weight) of bacteria. Absorbed and unabsorbed antibodies were checked as potential inhibitors of binding of ^{125}I-collagen to *S. aureus* Cowan. *Symbols*: unabsorbed antibodies, 200 μg/ml (O), antibodies absorbed with cells of *S. aureus* Cowan (□, 25 μg/ml), antibodies absorbed with cells of *S. aureus* Newman (△, 26 μg/ml).
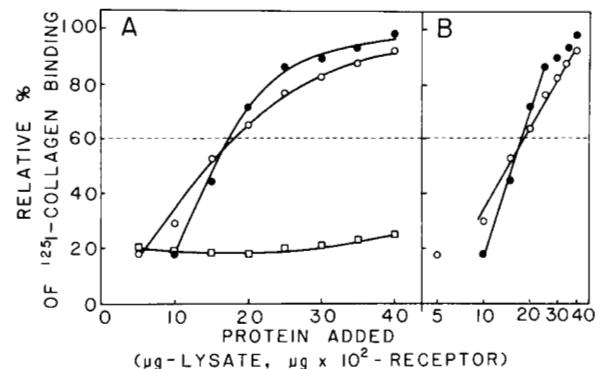
FIG. 4. **Neutralization of inhibitory activity of polyclonal anti-*S. aureus* antibodies with lysates of homologous and heterologous bacteria.** Immune anti-*S. aureus* Cowan Fab fragments (60 μl, *i.e.* 12 μg, the amount that reduces collagen binding by 80%, see Fig. 2) were incubated for 1 h at 37 °C with the indicated amounts of lysostaphin lysates of strain *S. aureus* Cowan (O) or Newman (□) or the purified *S. aureus* Cowan collagen receptor (●). The absorbed antibodies were tested in the neutralization assay as indicated in the legend to Fig. 2. *Panel A* presents the data in the linear scale; *panel B*, the same data as a semilogarithmic plot. *Dashed line* indicates the concentration of material required to restore half of the inhibition caused by the addition of the indicated amount of antibodies (40% of inhibition, *i.e.* 60% of binding). This amount of material is defined as one receptor unit.

inhibit the binding of ^{125}I-collagen to intact bacteria. The results (Fig. 4) were consistent with those presented in Fig. 3 in that the inhibitory activity of anti-Cowan antibodies was neutralized only by preincubation of the Fab fragments with lysate of strain Cowan but not with lysate of strain Newman. The observed neutralization of antibody activity depended on the concentration of the lysates (Fig. 4A), and a linear relationship between neutralization activity and amount of lysate added was observed when the data were plotted on a semilogarithmic scale (Fig. 4B).

Based on these observations an assay was designed for quantitating solubilized collagen receptors. We here assumed that the component(s) responsible for the neutralizing activity is identical to the collagen receptor. In this assay, details of which are described under "Materials and Methods," predetermined amounts of anti-Cowan antibodies which cause 80% inhibition of ^{125}I-collagen binding to bacteria are initially incubated with varying amounts of solubilized receptor. Subsequently cells of *S. aureus* Cowan and ^{125}I-collagen are added. The incubation is continued, and the amount of labeled ligand bound to the bacteria is determined. An amount of receptor

FIG. 2. **Inhibition of ^{125}I-collagen binding to cells of *S. aureus* Cowan by antibodies.** Bacteria, 1×10^8 cells, were incubated with the indicated amounts of Fab fragments (200 μg/ml) for 1 h prior to incubation with ^{125}I-collagen for 30 min. The amount of bound ^{125}I-collagen was determined as described under "Materials and Methods." The results are expressed as a percentage relative to binding in the absence of antibodies. *Symbols*: anti-*S. aureus* Cowan preimmune (△) and immune (O); anti-*S. aureus* Newman immune (□) antibodies.

neutralizing half of the inhibitory activity of the antibodies has been arbitrarily defined as one receptor unit.

Purification of the Collagen Receptor

Solubilization of the Receptors—The protocol used for solubilization of collagen receptors from staphylococcal cells involves digestion of bacteria with lysostaphin in the presence of protease inhibitors as described previously for solubilization of fibronectin receptors of *S. aureus* strain Newman (Fröman *et al.*, 1987). Preliminary experiments showed that a brief sonication of bacteria (3 min) substantially improves the yield of collagen receptors, and this step was therefore included in the protocol.

Attempts to absorb the collagen receptor on a Sepharose column substituted with type I or II collagen or gelatin were unsuccessful. This result is perhaps not surprising in view of the inability of solubilized receptors to inhibit the binding of ^{125}I-collagen to bacteria.

We have routinely used heat-treated bacteria as a source of the collagen receptor. Since Holderbaum *et al.* (1986) pointed out that heat treatment may lower the affinity of collagen receptor for its ligand, we attempted to isolate the receptors from bacteria which were not heat-treated. However, this change did not result in a binding of receptors to the collagen-Sepharose column nor did lowering the ionic strength of the buffer in which the solubilized material was applied to the column. Affinity chromatography was therefore not a viable method of collagen receptor purification, and alternative methods had to be sought. A three-step procedure was developed.

Ion Exchange Chromatography—Practically all protein components in the bacterial lysate were adsorbed on a column of DEAE-Sephacel when applied at neutral pH and low ionic strength. The adsorbed material eluted from the column with a NaCl gradient as a series of peaks (Fig. 5). The main portion of receptor activity was eluted with less than 0.2 M NaCl, although some residual activity was still detected in fractions eluted with higher salt concentrations. Ion exchange chromatography provided an excellent initial purification step, accompanied by high recovery (75%) of the active material (Table I). Peaks containing appreciable receptor activity, *i.e.* pools Ia, Ib, and Ic, were combined, dialyzed to water, and freeze-dried.

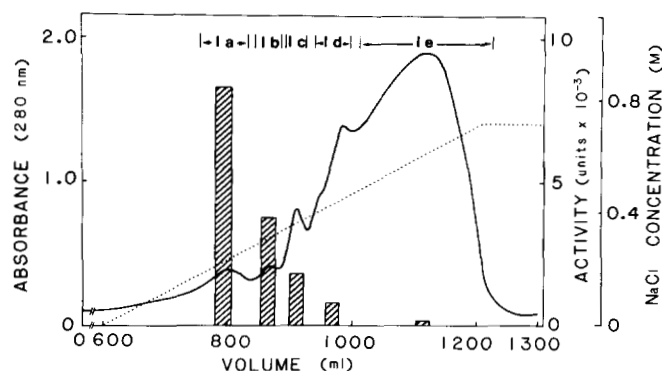*Ammonium Sulfate Precipitation*—Electrophoretic analysis

FIG. 5. **DEAE-Sephacel chromatography of *S. aureus* lysate.** Lysate (550 ml) of *S. aureus* Cowan obtained after digestion of 50 g of bacterial cells dialyzed to the starting buffer was passed at a rate of 25 ml/h through a column (3.3 × 30 cm) of DEAE-Sephacel equilibrated with 50 mM Tris-HCl, pH 7.4. The bound material was eluted with NaCl gradient (0–0.7 M). *Hatched bars* indicate units of receptor activity in pooled fractions after dialysis to water. Pools Ia through Ic were combined, dialyzed to distilled water, and freeze-dried.

of material purified by ion exchange chromatography (Fig. 7, *lane g*) revealed substantial amounts of high molecular weight material which migrated as smeary bands. This material could be precipitated with 60% ammonium sulfate, while the collagen receptor activity remained in solution. Practically all the receptor activity could be recovered at this purification step (Table I). After extensive dialysis the material not precipitable with 60% ammonium sulfate (pool IIa) was freeze-dried and subjected to the next step of purification.

Gel Filtration—Further purification of the collagen receptor was achieved by gel filtration chromatography on a column of Sephacryl S-200 equilibrated with 6 M guanidinium hydrochloride supplemented with 0.1% *n*-octyl β-D-glucopyranoside. As shown in Fig. 6 material monitored by absorbance at 280 nm appeared as a series of peaks, while receptor activity was limited to the first and second peaks. The first peak, pool IIIa, contained one protein (M_r 135,000) as shown by PAGE in SDS (Fig. 7, *lane j*). The second peak, pool IIIb, contained components of apparent lower molecular weight in addition to the 135-kDa protein (Fig. 7, *lane k*). Rechromatography of pool IIIb under the same conditions resulted in a separation of the high and the low molecular weight materials. Collagen receptor activity is limited only to the high molecular weight component (135,000). The gel chromatography step of purification resulted in loss of about half of the active material, but overall recovery of receptor activity was 33%. The purification procedure resulted in a 179 times increase of specific activity (Table I). These data suggest that a 135-kDa protein constitutes the main component in the lysate which neutralizes the inhibitory activity of antibodies and therefore presumably represents a collagen receptor. Support for this conclusion was obtained in an experiment where samples from different stages of purification were separated by electrophoresis (as in Fig. 7) and electroblotted into Immobilon-P membrane and then probed with anti-Cowan antibodies absorbed with cells of strain Newman. In a parallel experiment electroblotted material was directly stained with Amido Black to ensure that the protein bands in the gel are indeed transferred onto the membrane (data not shown). The results (Fig. 8) clearly show that only one major protein band in the whole lysate is recognized by the inhibitor antibodies. The molecular weight of this material corresponds to that of the purified receptor. Moreover, the occurrence of this band is limited to the pools which contain receptor activity.

Furthermore, the immunoreactive 135-kDa protein is not present in lysate generated from the collagen receptor-negative strain Newman nor do antibodies raised against strain Newman react with the 135-kDa protein in lysate of strain Cowan, when used in Western blot analysis (data not shown).

Preliminary Characterization of the Receptor Protein

Digestion of the receptor material with chymotrypsin, trypsin, V-8 protease, or thermolysin resulted in degradation of the receptor (Fig. 9, *lane c*) and complete loss of its activity in the antibody neutralization assay (data not shown). In contrast, this material was resistant to digestion by peptidoglycan degrading enzymes. Digestion with lysozyme, mutanolysin, or *Streptomyces globisporus* *N*-acetylmuramidase did not cause any significant loss of activity. The material remained virtually intact, although after prolonged digestion (18 h) with these three enzymes a minor band with M_r of 115,000 appeared in addition to the main 135-kDa band (Fig. 9, *lane b*). These data suggest that the epitope which the inhibitory antibodies recognize is of protein nature.

TABLE I

Purification of the collagen receptor from S. aureus Cowan 1

This table summarizes the data presented in Figs. 5 and 6. The data presented in the table refer to the amount of lysate obtained from 50 g (wet weight) of bacteria. More than 90% of receptor activity was released from bacterial cells under the conditions employed.

Pool	Volume	Protein[a]	Protein	One-unit volume	Activity	Specific activity	Yield	Purification
	$\mu l \times 10^{-3}$	$\mu g/ml$	$\mu g/pool$	μl	units/pool	units/mg	%	
Lysate	550	450	247,500	67	8,209	33.2	100	1.0
Ia	162	35	5,670	44	3,682	649.4	44.9	19.6
Ib	62	205	12,710	37	1,676	131.9	20.4	4.0
Ic	94	211	19,834	120	783	39.5	9.5	1.2
Id	124	200	24,800	281	441	17.8	5.4	0.5
Ie	396	600	237,600	>300	<1,320	<5.5	<1.6.1	<0.2
Ia/b/c	318	120	38,160	52	6,115	160.0	74.5	4.8
IIa	23	590	13,570	>300	<77	<5.7	<0.9	<0.2
IIb	125	145	18,125	22	5,682	313.4	69.2	9.4
IIIa	13	24	312	7	1,857	5,952.3	22.6	179.3
IIIb	36	35	1,260	28	1,286	1,020.4	15.7	30.7
IIIc	75	70	5,250	122	615	117.1	7.5	3.5
IIId	40	240	9,600	>300	<133	<13.8	<1.6	<0.4
IIIb/c, rerun	12	22	264	15	800	3,030.3	9.7	91.3

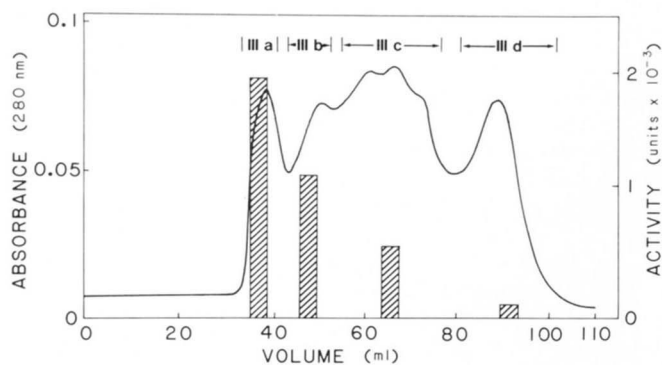[a] Determined according to Bradford (1976) using human IgG as a standard.

FIG. 6. **Sephacryl S-200 chromatography.** Material not precipitable with 60% ammonium sulfate was dialyzed to water, freeze-dried, and dissolved in a small volume of 2 M guanidinium chloride containing 0.1% *n*-octyl β-D-glucopyranoside and passed at the rate of 20 ml/h through a column of Sephacryl S-200 (2 × 150 cm) equilibrated with the same buffer. Typically fractions eluted in the first peak contained chromatographically pure material and most of the receptor activity. Receptors present in pools IIIb and IIIc were rechromatographed at the same conditions.
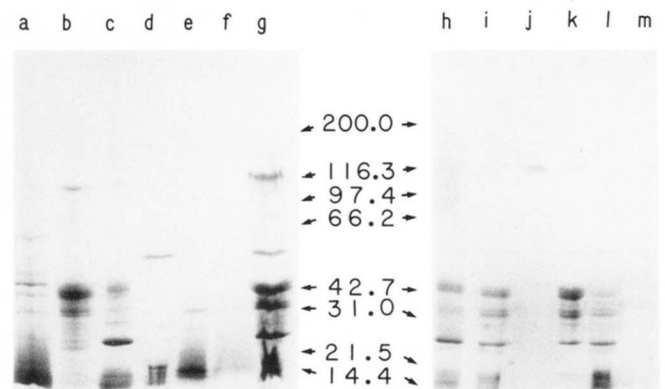

FIG. 7. **Polyacrylamide gel electrophoresis (SDS-PAGE, reducing conditions) of fractions at different purification steps.** Materials from the different purification steps were subjected to electrophoresis in 4.5–10% polyacrylamide gel; proteins in the gel were stained with Coomassie Brilliant Blue R-250. *Lane a*, untreated lysate of *S. aureus* Cowan; *lanes b–f*, fractions from DEAE-Sephacel chromatography: *b*, Ia; *c*, Ib; *d*, Ic; *e*, Id; *f*, Ie; *lane g*, combined pool of Ia, Ib, and Ic; *lane h*, material precipitated with 60% ammonium sulfate; *lane i*, material not precipitable with 60% ammonium sulfate; *lanes j–m*, pools from gel filtration chromatography: *j*, IIIa; *k*, IIIb; *l*, IIIc; *m*, IIId. *Numbers* and *arrows* in the middle of the figure indicate molecular weights (× 10^{-3}) and migration distances of standard proteins.

Characterization of Anti-receptor Antibodies

The 135-kDa material has been injected into a rabbit to generate polyclonal antibodies. Fab fragments of generated anti-receptor antibodies have subsequently been used in a series of experiments analogous to these performed with anti-Cowan antibodies. Anti-receptor antibodies inhibited binding of ^{125}I-collagen to bacteria, and their titer was approximately 2 times higher compared with that of anti-Cowan antibodies (data not shown). The inhibitory effect of these antibodies was abolished by absorption with collagen receptor-positive but nor receptor-negative bacteria (data not shown). A lysate of a collagen receptor-positive strain as well as the purified collagen receptor neutralized the inhibitory effect of the anti-receptor antibodies. The inhibitory pattern followed that observed with anti-Cowan antibodies (Fig. 4) and was linear when plotted semilogarithmically. In general, activity of anti-receptor antibodies did not substantially differ from the activity of anti-Cowan antibodies preabsorbed with cells of collagen receptor-negative bacteria. Also the pattern of immunostaining of Western blots with anti-receptor antibodies was analogous to that presented in Fig. 8 for staining with anti-Cowan antibodies.

In addition to polyclonal anti-receptor antibodies monoclonal antibodies have also been raised. Of several clones recognizing purified receptor some interfered with ^{125}I-collagen binding to bacteria (data not shown), which indicates that the receptor molecule has a domain structure where a certain domain is responsible for collagen binding. However, we have so far not been able to use the blocking monoclonal antibodies in Western blots.

DISCUSSION

We have previously shown that a strain of *S. aureus* Cowan 1 binds ^{125}I-collagen in a time-dependent reversible reaction. The bacterial cells could be saturated with labeled collagen, and an analysis of the binding data by Scatchard plot suggested that a maximum of 3×10^4 collagen type II molecules could be bound per cell and that the apparent K_d of the interaction was 10^{-7} M. Studies on the specificity of collagen
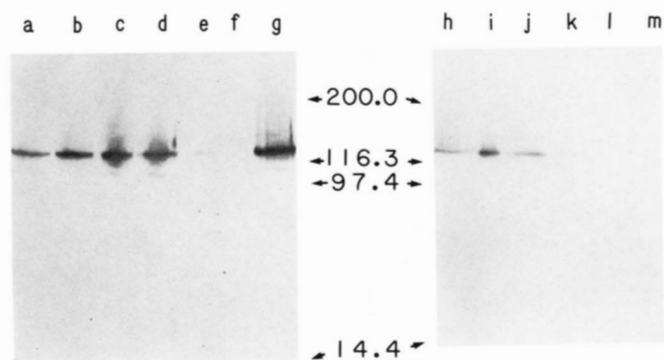
a b c d e f g   h i j k l m

FIG. 8. **Detection of putative collagen with antibodies.** Fractions from various purification steps (as in the legend to Fig. 7) after separation in the polyacrylamide gel were electroblotted into Immobilon-P membrane (Millipore, Bedford, MA) and probed with anti-*S. aureus* Cowan antibodies absorbed with cells of *S. aureus* Newman (see Fig. 3) (50 μg of Fab fragments in 100 ml of solution). The membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Bio-Rad) followed by the appropriate substrate.
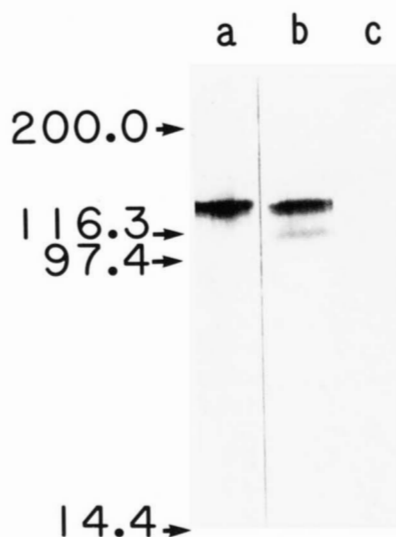
a   b   c

FIG. 9. **Digestion of purified collagen receptor with muralytic and proteolytic enzymes.** Purified receptor, 50 μg, was digested for 18 h with 5 μg of different enzymes. After inactivation of the enzymes (89 °C, 20 min) in the undigested and digested materials were separated by electrophoresis, blotted, and processed as described in the legend to Fig. 8. *Lane a*, untreated receptor; *lane b*, receptor digested with the mixture of lysozyme, *N*-acetylmuramidase, and mutanolysin; *lane c*, receptor digested with trypsin. Data presenting the digestions with other proteolytic enzymes are not included.

binding to *S. aureus* cells demonstrated that the receptor does not recognize noncollagenous proteins but all the collagen types tested. Furthermore, isolated collagen a chains, collagen fragments, and synthetic collagen analogs inhibit the binding of ^{125}I-labeled type II collagen to bacteria (Speziale *et al.*, 1986). These data suggested that the staphylococcal receptor recognizes a fairly simple structure in the collagen molecule.

Since the collagen receptor appears to be a major staphylococcal cell surface protein it is possible that the density of the receptor on the bacterial cell surface is high enough to allow a collagen ligand molecule to simultaneously interact with several receptor molecules. The affinity of such an interaction is an exponential function of the number of interactions. The previously measured K_d (10^{-7} M) would, if it reflects multiple receptors interacting with one ligand molecule, indicate that an individual receptor-ligand interaction may ex-

hibit a fairly modest affinity. In light of these observations it is perhaps not surprising that solubilized collagen receptors do not inhibit the binding of ^{125}I-collagen to bacterial cells or absorb to an affinity matrix containing immobilized collagen. The affinity of an individual receptor molecule may simply be too low to allow us to detect an interaction with these methods. Consequently, could we design experiments where several receptors were simultaneously available to bind one collagen molecule it should be possible to demonstrate an interaction with reasonable affinity. In fact, preliminary experiments have shown that polystyrene beads coated with isolated collagen receptors bind ^{125}I-collagen in a reaction that resembles that between staphylococcal cells and collagen.[3]

It is also possible that the conformation of the solubilized receptor differs from that of a receptor on the surface of bacteria. Changes in the tertiary structure of a protein may directly affect conformation of the collagen ligand binding site and hence the affinity for the ligand. Similar changes in the antigenic epitopes in fibrinogen molecule following its enzymatic digestion were reported by others (Cierniewski and Budzynski, 1987; Nagy *et al.*, 1985).

Another consequence of the apparent low affinity that the solubilized staphylococcal collagen receptor has for its ligand is that direct binding methods are essentially useless in the identification, quantification, and isolation of the collagen receptor. Indirect methods had to be employed. In this study we adopted an immunological assay, which previously has been used to identify cell surface molecules involved in eukaryotic cell-cell adhesion (Müller and Gerisch, 1978; Ocklind and Öbrink, 1982; Urushihara and Takeichi, 1980). In this assay solubilized proteins without apparent biological activity are used to neutralize the inhibitory effect of the crude antibodies. With the help of this assay we have isolated a putative collagen receptor protein of an apparent M_r of 135,000. This protein has been used to generate polyclonal as well as monoclonal antibodies which inhibit the binding of collagen to bacteria. The active antibodies also specifically recognize the 135-kDa protein when used in Western blot assay demonstrating that the epitopes recognized by the inhibitory antibodies are located on the 135-kDa protein. Although a direct binding of the 135-kDa protein to collagen has not been demonstrated in this study it should be pointed out that this protein is present on the surface of a collagen receptor-positive strain but absent on a collagen receptor-negative strain. Consequently we propose that the 135-kDa protein is a staphylococcal collagen receptor.

In addition to *S. aureus*, bacteria of some other species were reported to bind collagen, namely streptococci (Kostrzynska *et al.*, 1989; Mamo *et al.*, 1987), *Salmonella* sp. (González *et al.*, 1988), and *Escherichia coli* (Ljungh and Wadström, 1988; Westerlund *et al.*, 1989). In these cases, however, the corresponding receptors have not been identified, isolated, or characterized. Also eukaryotic cells have been shown to bind collagen, and some of the receptors involved were characterized. A collagen receptor of the integrin type from fibrosarcoma cells appears to recognize an Arg-Gly-Asp sequence in type I collagen (Dedhar *et al.*, 1987). A collagen binding protein from chondrocytes has been also isolated, cloned and sequenced (Pilar Fernandez *et al.*, 1988). Collagen binding proteins have been identified or isolated also from other cells (Santoro *et al.*, 1988; Sugrue, 1987; Velge *et al.*, 1988; Wayner and Carter, 1987). Of special interest in this context is a collagen receptor present on hepatocytes which binds collagen with a specificity similar to that of the staphylococcal receptor

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[3] L. M. Switalski, P. Speziale, and M. Höök, manuscript in preparation.

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(Rubin *et al.*, 1981). However, this receptor has not yet been isolated and characterized. The relationship between these identified collagen binding proteins and *S. aureus* collagen receptor reported here is unclear.

The collagen receptors of *S. aureus* have in preliminary experiments been shown to mediate the attachment of bacteria to collagen-coated surfaces.[3] Since *S. aureus* strains are the leading cause of osteomyelitis and infectious arthritis, where bacteria colonize collagen-rich tissues such as cartilage and bone, it is possible that the binding of bacteria to collagen represents a mechanism of bacterial adherence to these tissues. Preliminary data from our group (Voytek *et al.*, 1988) and others (Holderbaum *et al.*, 1987) are suggestive of the relationship between the ability of staphylococci to colonize collagen-rich tissues and the expression of collagen receptors. We are currently examining this hypothesis.

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