Collagen Binding to *Staphylococcus aureus*

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Staphylococcus aureus can bind soluble collagen in a specific, saturable manner. We have previously shown that some variability exists in the degree of collagen binding between different strains of heat-killed, formaldehyde-fixed *S. aureus* which are commercially available as immunologic reagents. The present study demonstrates that live *S. aureus* of the Cowan 1 strain binds amounts of collagen per organism equivalent to those demonstrated previously in heat-killed, formaldehyde-fixed bacteria but has an affinity over 100 times greater, with K_d values of 9.7×10^{-11} M and 4.3×10^{-8} M for live and heat-killed organisms, respectively. Studies were also carried out with *S. aureus* killed by ionizing radiation, since this method of killing the organism seemed less likely to alter the binding moieties on the surface than did heat killing. Bacteria killed by exposure to gamma radiation bound collagen in a manner essentially indistinguishable from that of live organisms. Binding of collagen to irradiated cells of the Cowan 1 strain was rapid, with equilibrium reached by 30 min at 22°C, and was fully reversible. The binding was not inhibited by fibronectin, fibrinogen, C1q, or immunoglobulin G, suggesting a binding site for collagen distinct from those for these proteins. Collagen binding was virtually eliminated in trypsin-treated organisms, indicating that the binding site has a protein component. Of four strains examined, Cowan 1 and *S. aureus* ATCC 25923 showed saturable, specific binding, while strains Woods and S4 showed a complete lack of binding. These results suggest that some strains of *S. aureus* contain high-affinity binding sites for collagen. While the number of binding sites per bacterium varied sixfold in the two collagen-binding strains, the apparent affinity was similar. The ability of *S. aureus* to bind collagen with high affinity may provide a mechanism for bacterial adhesion to host tissue and thereby play a role in the invasive characteristics of this organism.

Staphylococcus aureus has been shown to bind specifically to an increasing variety of mammalian proteins. Plasma proteins that have been shown to bind specifically to *S. aureus* include fibrinogen (8), immunoglobulin G (IgG) (3, 6), fibronectin (7, 11, 19), and C1q (17). This organism has also been demonstrated to bind to elements of the extracellular matrix, including laminin (9), type IV collagen (22), fibronectin (7, 11, 19), and type I procollagen and collagen (4). It has been suggested that the presence of specific surface binding sites for extracellular matrix components might enable this organism to adhere to specific proteins located in tissues and thereby serve as an adhesion mechanism by which infective foci could become established (12). The ability of *S. aureus* to bind collagen specifically and with high affinity may therefore be an important characteristic associated with the virulence of this organism.

Our original observations of collagen binding to *S. aureus* and the measurements of number and affinity of these sites were conducted with commercial preparations of *S. aureus* (4). These preparations were intended primarily for use as immunochemical reagents for the adsorption of IgG. Since heat killing and formaldehyde fixation of these strains may alter the binding kinetics of the collagen from those seen in live organisms or strains killed by a less disruptive method, the experiments described in this report were undertaken. We report here the results of collagen binding to four strains of *S. aureus*. Binding kinetics studies were conducted on live as well as radiation-killed organisms. These experiments show that while the total amount of collagen bound per organism is similar in live and irradiated bacteria when compared with heat-killed and fixed bacteria, live cultures and irradiated cultures bind collagen with a markedly increased affinity.

MATERIALS AND METHODS

Purified proteins. Soluble calf skin collagen was obtained from U.S. Biochemicals, Cleveland, Ohio. Rabbit plasma fibronectin was purified from frozen EDTA-treated rabbit plasma (Pel-Freez, Rogers, Ariz.) by the method of Miekka et al. (10). IgG was isolated from nonimmune goat serum by Na_2SO_4 precipitation and DEAE-cellulose chromatography (18). Human C1q was purchased from Calbiochem-Behring, La Jolla, Calif. Human fibrinogen was provided by J. R. Shainoff of the Thrombosis Section of Cleveland Clinic (15). Bovine pancreatic trypsin, twice crystallized, was obtained from Worthington Diagnostics, Freehold, N.J. Bovine serum albumin was purchased from Sigma Chemical Co., St. Louis, Mo.

S. aureus **strains.** The following strains were purchased from the American Type Culture Collection, Rockville, Md.: 10832 (Woods), 11631 (S4), 25923, and 12598 (Cowan 1). After reconstitution, the strains were kept frozen at −20°C in sheep blood. Individual strains were cultured on blood agar overnight at 35°C and subcultured in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for 24 h at 35°C. Bacterial suspensions were centrifuged at 2,500 × *g* for 15 min at room temperature, and the growth medium was decanted and replaced with 0.05 M Tris–0.15 M NaCl (pH 7.5). The number of organisms per unit volume was estimated in all samples by a turbidimetric method (16).
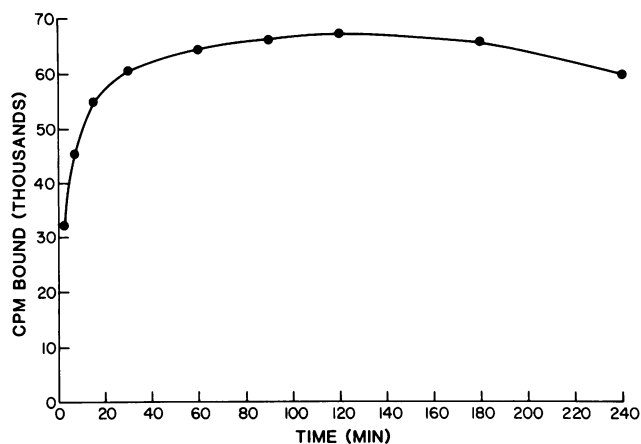
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FIG. 1. Time course of [^{125}I]collagen binding to *S. aureus*. The incubation mixture contained 250 ng of [^{125}I]collagen and 10^9 irradiated Cowan 1 cells per ml. Datum points have been corrected for nonspecific binding and represent the mean of triplicate samples.

Irradiation of bacterial cultures. A log dose-lethality curve was determined for the Cowan 1 strain in buffer suspension. Aliquots of bacterial suspensions were exposed to gamma radiation in a Mark I irradiator (J. L. Shepherd Associates, Glendale, Calif.) with a ^{137}Cs source and samples in a position to receive 1,300 rads/min. At increasing time intervals, samples were removed, and viable cells were counted by replicate plate counts. Equal aliquots of nonirradiated cells were counted as controls. Percent lethality was determined by the following calculation: [1 − (CFU$_{irr}$/CFU$_{cont}$)] × 100, where CFU$_{irr}$/CFU$_{cont}$ is the ratio of CFU of irradiated samples per ml to CFU of control samples per ml. The 50% lethal dose was calculated to be approximately 4 × 10^3 rads. Over 99% lethality was achieved at 3 × 10^4 rads. Routine irradiation of cultures was carried out by exposure of washed cells suspended in Tris-NaCl buffer to 10^5 rads.

Preparation of [^{125}I]collagen. Collagen was radioiodinated by the chloramine-T method (20) with Na^{125}I (17 Ci/mmol; New England Nuclear Corp., Boston, Mass.). Iodinated collagen was separated from free ^{125}I by gel filtration on Bio-Gel P-2 in Tris-NaCl buffer (pH 7.5). Protein-bound radioactivity was determined by 10% trichloroacetic acid precipitation of the iodinated collagen in the presence of 50% fetal calf serum. Typical preparations showed a specific radioactivity of approximately 7,000 cpm/ng of collagen. In most experiments, radiolabeled collagen was diluted prior to use to approximately 3,000 cpm/ng with unlabeled collagen (final concentration, 17 μg of collagen per ml). Labeled collagen was stored at 4°C and used within 4 days of radioiodination.

Time course experiment. Irradiated Cowan 1 cells were added to Tris-NaCl buffer (pH 7.5) containing 0.1% serum albumin to a final concentration of 10^9 cells per ml. Radioiodinated collagen was added at a concentration of 250 ng/ml. Nonspecific binding was determined in parallel by the addition of 250 μg of unlabeled calf skin collagen per ml prior to radioligand addition. Incubation was carried out at room temperature for intervals from 25 to 240 min with constant agitation. At the designated intervals, triplicate 1-ml samples were removed from each tube and centrifuged at 13,500 × *g* for 5 min. Supernatants were decanted, and [^{125}I]collagen bound to the bacterial pellets was measured by cutting the bottom from each tube and placing the pellets in tubes for

gamma counting. Previous experiments showed that washing of the bacterial pellets did not reduce the level of background radioactivity or nonspecifically bound radioactivity (4).

Reversibility experiment. In this experiment, the [^{125}I]collagen was not diluted to lower specific activity. Total-binding and nonspecific-binding samples were prepared as in the experiment described above. After 60 min of preincubation in the presence of 250 ng of [^{125}I]collagen per ml, unlabeled collagen was added to the total-binding tube to a concentration of 250 μg/ml. Samples were removed from 0 to 240 min as described above, with nonspecific binding corrected by subtraction.

Saturation binding experiments. These experiments were conducted by serial dilution of buffer containing 1,000 ng of [^{125}I]collagen per ml at the highest concentration to approximately 1 ng/ml at the lowest. Nonspecific binding was measured for each collagen concentration by replacing albumin (1 mg/ml) with unlabeled calf skin collagen (1 mg/ml). Bacteria were added to a concentration of 10^9 organisms per ml, and incubations were carried out for 90 min at either room temperature or 4°C with constant mixing. Triplicate samples were removed at each total- and nonspecific-binding concentration. Bacterial pellets were obtained and counted as described above. In experiments with heat-killed organisms, cultures were heat killed at 80°C for 5 min and fixed in 1.5% formaldehyde (5).

Inhibition experiments. Irradiated Cowan 1 cells were suspended in Tris-NaCl buffer (pH 7.5) containing 1 mg of albumin and 10 μg of rabbit plasma fibronectin, human C1q, human fibrinogen, or goat IgG per ml. Preincubations were carried out for 2 h at room temperature with agitation. Following preincubation, [^{125}I]collagen was added at 2.5, 25, or 250 ng/ml. For nonspecific binding, a set of samples containing both 250 μg of nonlabeled collagen per ml and [^{125}I]collagen was incubated in the same manner. After 90 min, triplicate samples of bacterial suspensions were assayed for specifically bound collagen as described above.

Trypsin treatment of *S. aureus*. Cowan 1 cells were suspended in Tris-NaCl buffer (pH 7.5) containing 1 mg of trypsin per ml for 30 min at 37°C (22). After digestion, suspended Cowan 1 cells were washed twice in Tris-NaCl
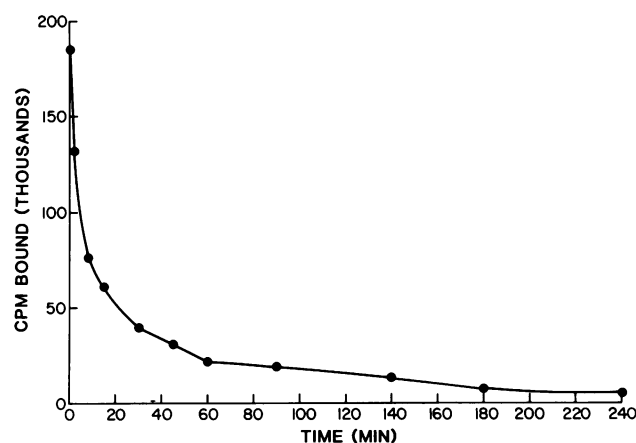

FIG. 2. Reversibility of collagen binding at room temperature. Datum points represent the mean of triplicate determinations corrected for nonspecific binding. At time zero, a 1,000-fold excess of unlabeled collagen was added to irradiated Cowan 1 cells which had been preincubated for 2 h with 250 ng of [^{125}I]collagen per ml.

buffer containing 1 mg of serum albumin per ml. Collagen was added at the same concentrations used in the inhibition experiment to cell suspensions containing 10^9 bacteria per ml.

RESULTS

Time course and reversibility. At room temperature, specific binding of collagen to irradiated Cowan 1 cells occurred rapidly, reaching one-half maximal levels within 3 min and approaching plateau levels by 30 min. After 3 and 4 h of incubation, the level of binding decreased to a small extent, indicative perhaps of some autolytic activity (Fig. 1); the fraction of radioactivity bound nonspecifically was constant and, with the exception of the 2.5-min time point, represented less than 10% of total binding. Binding was completely reversible at room temperature with the addition of a 1,000-fold excess of unlabeled collagen. The time required to reach 50% reversibility was approximately 5 min; reversibility was essentially complete by 4 h (Fig. 2).

Effect of irradiation and heat killing on saturation binding. Cowan 1 cells killed by gamma radiation bound collagen at room temperature in a manner similar to that of live cells (Fig. 3). Similar results were obtained at 4°C (data not shown). The saturation binding and K_d values of live and irradiated organisms were virtually the same. In contrast, heat-killed and fixed Cowan 1 cells bound much less collagen at room temperature than did either live or irradiated cells. The amount of collagen bound at 1,000 ng/ml by heat-killed cells was comparable to that bound at that concentration when immunoadsorbent *S. aureus* was used (4). The total calculated number of specific sites of binding for collagen to live and irradiated *S. aureus* at saturating concentrations in these experiments was approximately the same as that previously reported for immunoadsorbent *S. aureus* (4).

Since virtually all of the ligand was bound to the *S. aureus* at collagen concentrations of 1 to 8 ng/ml, Scatchard analysis with bound/free ligand ratios was not possible. As an alternative, the K_d for collagen binding to Cowan 1 cells was estimated by constructing a Hill plot (Fig. 4). The apparent
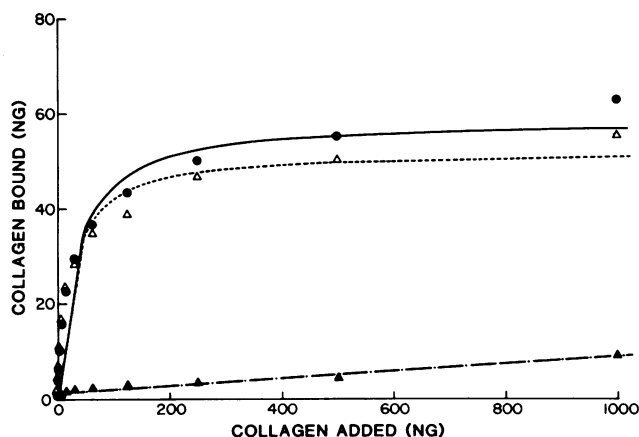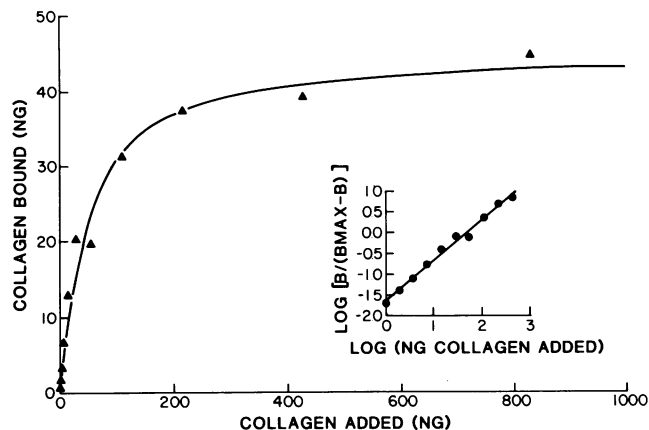

FIG. 4. Determination of K_d values by a Hill plot. The experimental conditions were the same as those in Fig. 3 (see Materials and Methods). The binding data were graphically represented as a Hill Plot (inset). The linear equation of the Hill plot is $y = 0.977x - 1.64$; $r = 0.995$, $K_d = x$ when $y = 0$.

K_d of binding was approximately 6×10^{-11} M. This agreed well with K_d values calculated by fitting the binding data to a hyperbola with the equation $y = (B_{max}x)/(K_d + x)$, where y is nanograms of collagen bound to 10^9 organisms and x is the collagen concentration. Consequently, this curve-fitting procedure was used in subsequent studies for the determination of affinity constants.

Collagen binding to various *S. aureus* strains. Of the four strains tested, only two, Cowan 1 and ATCC 25923, bound collagen. Strains Woods and S4 showed no specific binding at any concentration tested (Fig. 5). While Cowan 1 bound approximately six times more collagen per organism than did ATCC 25923, the affinity constants for the two positive strains were within 40% of each other, demonstrating similarity in binding affinity, with an apparent large difference in capacity (Table 1). We observed aggregation in the two strains which bound collagen. Neither S4 nor Woods formed aggregates, even at the 1-mg/ml collagen concentration.
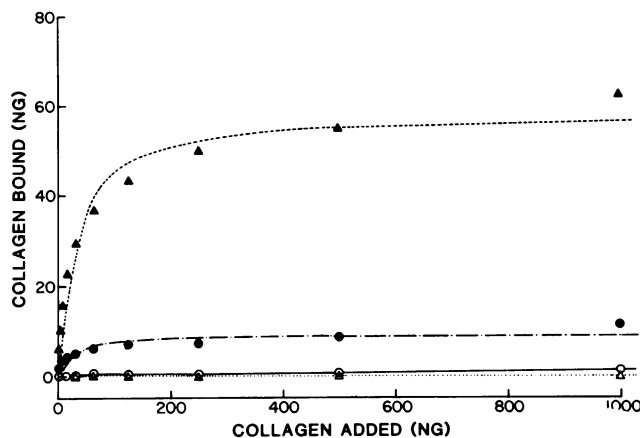

FIG. 3. Binding kinetics experiment on live ($\triangle$), irradiated ($\bullet$), and heat-killed, fixed ($\blacktriangle$) Cowan 1 cells. Datum points represent the mean of triplicate determinations of specific binding to 10^9 cells. Standard deviations for these data were within 5% of mean values for all points. The curves were fitted to the data by nonlinear regression with the equation $y = (B_{max}x)/K_d + x)$, where y is nanograms of collagen bound to 10^9 organisms and x is the collagen concentration.

FIG. 5. Binding of collagen to 10^9 irradiated Cowan 1 ($\blacktriangle$), ATCC 25923 ($\bullet$), S4 ($\bigcirc$), and Woods ($\triangle$) cells. Points represent the mean of triplicate samples. Note that the abscissa is below the zero point on the y axis. See Table 1 for K_d and binding-site values, which were calculated by previously described nonlinear curve-fitting procedures.
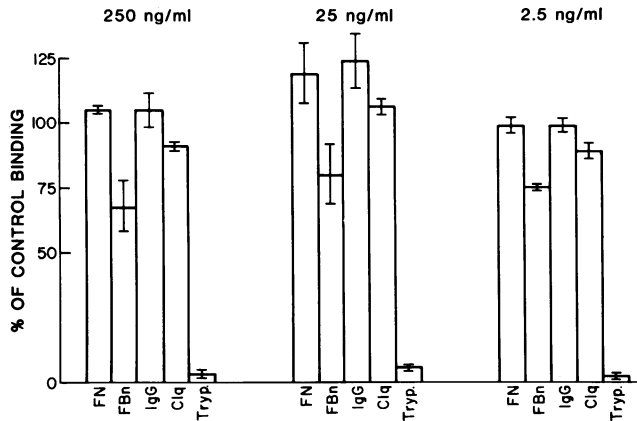
FIG. 6. Binding of collagen to *S. aureus* in the presence of the potential inhibitors fibronectin (FN), fibrinogen (FBn), IgG, and Clq. The effect of trypsin pretreatment of Cowan 1 cells is also shown (Tryp.). Collagen concentrations are shown over each group. Bars represent the mean ± standard deviation of three samples. 100% binding equals the amount of collagen bound at each concentration in the absence of an inhibitor. All values were corrected for nonspecific binding.

Cowan 1 formed aggregates at concentrations of collagen as low as 62.5 ng/ml. Aggregates formed in ATCC 25923 only in the nonspecific-binding samples in which the collagen concentration was 1 mg/ml. These results suggest that agglutination may be related to the number of collagen-binding sites present on the bacterial surface.

The ability to bind collagen was not retained upon storage. When irradiated Cowan 1 cultures were stored for 7 days at 4°C in Tris-NaCl buffer, the degree of binding was reduced to a level barely above nonspecific-binding levels, suggesting storage lability. Lability of the binding moiety was also suggested by the slight decrease in time-related binding at periods greater than 2 h (Fig. 1).

Inhibition of collagen binding by plasma proteins and trypsin sensitivity. Of the potential inhibitors of binding tested, only fibrinogen was capable of significant inhibition at all collagen concentrations tested (Fig. 6). The level of fibrinogen during preincubation and during ligand binding was in an approximate 12-fold molar excess with respect to the maximal collagen concentration. This suggests that the 30% inhibition of collagen binding by fibrinogen is probably due to a steric rather than a specific inhibition of binding.

Collagen binding at all levels tested was essentially extinguished by prior treatment of the Cowan 1 strain with

TABLE 1. Collagen binding to *S. aureus* strains[a]

Strain	No. of binding sites/bacterium	K_d (10^{-11} M)
Cowan 1	117	9.74
ATCC 25923	19	6.33
S4	0	
Woods	0	

[a] The binding-site and K_d calculations were based on a molecular weight of 3×10^5 for collagen. The binding-site data assume that each molecule of collagen binds to only a single binding site on the bacterial surface. The actual number of binding sites may be considerably higher if multiple occupation of sites by a single collagen molecule occurs. The K_d was calculated as the concentration at which one-half maximal binding occurs (see the text).

trypsin, suggesting that the integrity of the bacterial surface protein is required for collagen binding.

DISCUSSION

The presence of specific, high-affinity binding sites for collagen on some strains of *S. aureus* suggests that collagen-binding capacity may play a role in the tissue invasiveness of these bacteria by providing a mechanism whereby these cells might attach themselves to a tissue matrix. Since these studies demonstrate that collagen binding is not directly inhibited by certain plasma or matrix proteins, collagen probably adheres to a binding site distinct from those of the other proteins tested, raising the possibility that multiple-site binding interactions between the bacteria and different extracellular matrix components of the tissue occur. The collagen-binding site on *S. aureus* is presently not characterized to the same extent as the laminin (9)- or fibronectin (14)-binding sites. Studies on the isolation and characterization of the collagen-binding site are currently in progress.

Our findings with respect to trypsin sensitivity and aggregation suggest that the agglutination response to type IV collagen reported by Vercellotti et al. (22) may be related to the collagen-binding capacity of the Cowan 1 strain in a manner similar to the increased fibronectin-induced agglutination observed in *S. aureus* isolates containing higher numbers of fibronectin-binding sites (12). Further evidence supporting this suggestion comes from the finding of a strain-specific, graded response to the addition of aggregating agents (22). Our results also agree with those of Vercellotti et al. (22) with regard to the lack of collagen binding by the Woods strain. The present study confirms the inability of IgG to block collagen binding, as we previously reported (4). When the inability of soluble protein A to inhibit collagen binding or of protein A covalently linked to agarose to directly bind collagen (4) is considered, it appears likely that the presence of this IgG-binding protein on the Cowan 1 strain and its absence on the Woods strain are not related to the collagen-binding capacity of these strains.

While our observations are based on the binding of soluble collagen to *S. aureus*, it is important to note that for the binding to have physiological significance, the ability of this organism to bind solid-phase collagen must be shown. Two studies have demonstrated that this organism is capable of adhering to either type IV (22) or type II (1) collagen attached to a solid support.

The binding of collagen to *S. aureus* is totally reversible. This dissociability is in marked contrast with the *S. aureus*-fibronectin interaction, which has been shown to be essentially irreversible (13). That the collagen-*S. aureus* interaction can be rapidly and completely dissociated raises several possibilities which may be of potential importance in the treatment of some types of staphylococcal infections. If, for example, the infection were localized in an accessible site and collagen-*S. aureus* binding were the predominant adhesion mechanism, localized perfusion with a gelatin solution might be beneficial in dislocating matrix-bound *S. aureus* otherwise tightly bound to the collagen of the extracellular matrix of the infected tissue. Earlier studies carried out in this laboratory demonstrated the ability of gelatin to inhibit collagen binding (4). In the present study, gelatin was also found to directly inhibit native collagen binding, although not as efficiently as unlabeled native collagen (data not shown). It is also conceivable that agents could be developed which might effectively compete for the collagen-binding site, thus eliminating the need for introducing foreign proteins into the infection site.

The results of saturation binding experiments demonstrate several previously unreported aspects of the *S. aureus*-collagen interaction. First, it appears that treatment of the bacteria by the heat killing fixation method of Kessler (5) appreciably alters the kinetics of collagen binding. We previously reported the ability of commercial preparations of *S. aureus* to bind collagen (4). These immunologic probes were killed by 80°C exposure for 5 min and formaldehyde fixed. The current study shows that the total number of collagen molecules bound by *S. aureus* is not changed appreciably by heat killing. However, the affinity of binding is over 100-fold greater for live cells or cells killed by ionizing radiation as compared with previously determined affinity constants for binding to killed, fixed organisms, suggesting a heat-induced conformational change in the binding moiety rendered permanent by formaldehyde fixation.

Second, of the four strains tested, two bound collagen, and two did not. Of the two which showed positive binding (Cowan 1 and ATCC 25923), the affinity constants for binding were similar, while the total amount of collagen bound was markedly different. This suggests that the *S. aureus* moiety responsible for binding may be quantitatively rather than qualitatively different between strains. The strains which do not bind collagen will be potentially useful as negative controls in studies designed to isolate and characterize the binding site from these organisms. In view of the strain-to-strain variability in collagen binding, it will be important to quantify the ability of *S. aureus* clinical isolates from a variety of infectious diseases to bind collagen to assess the potential clinical importance of this binding characteristic.

Third, the lability of the binding site upon storage at 4°C is presumably due to a form of autolysis. While our studies did not expressly attempt to prevent binding-site degradation, they illustrate an important storage characteristic which suggests that in the absence of protease inhibition, freshly prepared samples are required for binding experiments of this type. This loss of binding upon storage is in contrast to the ability of some *S. aureus* strains to bind human epithelial cells, a trait which is reportedly stable upon storage (2).

These experiments demonstrate a specific, high-affinity interaction between collagen and some strains of *S. aureus*. This binding is rapid and reversible. The ability of this microorganism to adhere to collagen may have important implications both in understanding the pathogenesis of staphylococcal infections and in their treatment. Further characterization of the collagen-binding site will enable investigators to determine how this binding might interact with other binding capabilities for different matrix components and promote the adhesion of *S. aureus* to different tissues.

ACKNOWLEDGMENTS

ADDENDUM

Since the submission of this paper, Speziale et al. (P. Speziale, G. Raucci, L. Visai, L. M. Świtalski, R. Timpl, and M. Höök, J. Bacteriol. 167:77–81, 1986) have confirmed and expanded our original observation of collagen binding to heat-killed Cowan 1 *S. aureus* (4). The binding described by

these authors is reversible and partially inhibitable by fibrinogen, results which our present study also demonstrate. The binding that they found, however, shows markedly reduced affinity and increased capacity when compared with the binding that we found. This may be a function of the heat-kill method that they used, which may have substantially altered the kinetics of collagen binding to *S. aureus*, as suggested by our present results.

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