

CHARACTERIZATION OF THE FIBRINOGEN AND
FIBRIN ADHESIN OF STAPHYLOCOCCUS AUREUS

by

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ABSTRACT

The interaction between Staphylococcus aureus and cross-linked and non-cross-linked fibrin has been characterized and the cell wall component mediating fibrinogen induced clumping has been identified. Binding of S. aureus to fibrin and clumping of the cells in fibrinogen, appear to be mediated by the same cell wall component, called clumping factor (Clf).

The binding of S. aureus to fibrin was maximal at 37°C with a pH optima of 6-8 and was independent of divalent cations. The interaction between the two surfaces occurred within minutes and once bound, cells were difficult to remove. The fibrin-binding reaction was inhibited by 1M NaCl and 4M urea but not by any protein, glycoprotein, glycolipid, or any specific sugar tested. Heating the cells, treating them with protease, or acetylating amino groups abrogated their binding activities.

A glycoprotein, believed to be the Clf, has been partially purified from lysostaphin solubilized cell walls of S. aureus, Clf+ strains, by affinity chromatography on immobilized fibrinogen. The Clf active fraction bound to fibrinogen and eluted with 2M NaCl. Analysis of the fraction by gel electrophoresis revealed a single band, molecular weight approximately 90,000 daltons, which stained positively for protein and carbohydrate. This glycoprotein could not be identified in cell walls of a Clf negative strain of S. aureus. Immuno-electrophoretic analysis of the clumping factor fraction, showed that it formed a single precipitin line with anti- Clf+ S. aureus antiserum (anti-S. aureus-1) but not

with antiserum raised against the Clf- mutant. An assay was developed to quantitate the soluble Clf, and showed that the Clf absorbed or neutralized specific clumping inhibiting antibodies present in anti-S. aureus-1, that prevented fibrinogen induced clumping of S. aureus.

Other Clf- mutants of S. aureus and non-staphylococcal bacteria were examined for fibrin and fibrinogen binding and fibrinogen clumping activities. The majority of bacteria observed that could bind to fibrin and fibrinogen, could not clump in the presence of fibrinogen. A model is presented to describe adhesins that bind to fibrinogen but are unable to form cell-aggregates.

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INTRODUCTION

In many environments, the ability of a microorganism to selectively adhere to a surface is an essential step in the colonization of that area. Adherence to a solid surface may prevent the organism's removal by hydrodynamic or other physical forces and may assist in its acquisition of food particles that have adsorbed to the same surface (11,22,23,31,39,41,72,74).

Early studies of microorganisms in natural, aqueous environments showed them capable of attaching to a variety of solid materials; clay, sand, glass, cellulose, plastic, or other bacterial and eukaryotic cells (21,22,23,72). Many of the adherent organisms were found to possess a variety of specialized surface structures, implicated as the factors mediating binding (9,11,12,42,72).

Recently, attention has focused on the adherence of bacteria to mammalian tissues. The reader is referred to several excellent reviews on the subject (12,39,41,82,91,92). Organisms that populate the mammalian skin or mucosa of the respiratory, alimentary, or urogenital tracts, are constantly being challenged by defence mechanisms of the host. These mechanisms, be they a mucosal blanket and ciliary or peristaltic movements, can be largely surmounted by a microbe if it has the means for attachment. Most investigators in the field of adherence concur with the statement made by Gibbons, 1977, (39),

"The attachment of an organism to a host tissue therefore appears to be the first essential step for persistent colonization."

Selective nature of bacterial attachment to mammalian tissue

Increasing evidence indicates that microorganisms do not adhere to host tissue indiscriminately, but show a specificity for certain cell surfaces. Much of our present knowledge on the specificity of bacterial adherence in the mammalian host has resulted from studies of the oral ecosystem. This area is easily accessible and has received much attention because of the universal incidence of microbially induced dental diseases.

Gibbons and collaborators, (32,39,40,41,42,43,62), pioneered work in this area and have reached several conclusions:

- (i) Bacteria indigenous to the oral cavity adhere selectively to epithelium, to the tooth surface, or to other bacteria.
- (ii) The affinity of each bacterial species for a particular oral surface correlates positively with their natural occurrence at that site.

Streptococcus salivarius and Streptococcus mitis show a predilection for specific epithelial surfaces. S. salivarius prefers the dorsum of the tongue whereas S. mitis adheres to, and colonizes non-keratinized buccal mucosa (41,62). Other oral bacterial species adhere to the tooth pellicle or to bacterial cells, and form dental plaque (43). The acquired pellicle on the tooth surface is an organic film, adsorbed to the enamel and consists of salivary proteins, glycoproteins and immunoglobulins.

Streptococcus mutans and Streptococcus sanguis are examples of oral microorganisms that selectively adhere to the pellicle (40). The accumulation of bacteria in plaque also involves interactions of bacteria with cells of the same or dissimilar species. These adherent interactions appear to be mediated by substances that are either produced by the bacteria or are adsorbed to their cell surfaces from the surrounding milieu. Some examples

of these interbacterial aggregations are:

- (i) the "corn-cob formations" of Bacteroides matruchotii and oral streptococci, or Actinomyces naeslundii and S. sanguis (43).
- (ii) S. salivarius and Veillonella alcalescens (99).
- (iii) S. sanguis or S. mutans bound to Actinomyces viscosus (15,68).

Selective adherence was also observed with the indigenous microflora of the gastrointestinal tract of rodents and chickens; these microorganisms showed a preference for either secreting, or nonsecreting, keratinized cells (85,86).

Aly et al (3), found a high degree of specificity in the adherence of bacteria to nasal mucosal cells. Organisms most commonly found in the nose included species of Staphylococcus aureus and Staphylococcus epidermidis. These bacteria were found to adhere to nasal cells to a higher degree than organisms not normally present in the nose.

Adherence and pathogenicity

Although the pathogenicity of a microbe is not determined solely by its ability to bind to host tissue, it has been suggested that adherence is a necessary prerequisite to a number of microbial infections. Some examples are:

- (i) streptococcal pharyngitis by Streptococcus pyogenes (32)
- (ii) dental caries (40,43).
- (iii) gonorrhea (80,93)
- (iv) cholera (36,52,53). Strains of Vibrio cholera that could attach specifically to the brush borders of rabbit intestinal cells appeared to have an advantage

in colonizing that area over nonadherent strains

- (v) Diarrhea, caused by enterotoxigenic strains of Escherichia coli that could bind to porcine brush border cells. Strains of E. coli that failed to adhere to these cells did not cause the disease. (6,54).
- (vi) bacterial endocarditis (46,81). Organisms most frequently isolated from patients with bacterial endocarditis, are enterococci, viridans streptococci, staphylococci and Pseudomonas aeruginosa (46). These bacteria were also found to adhere more readily to damaged heart valves in vitro, than could other organisms not generally associated with the disease.

The first step in the initiation of these diseases appears to be the adherence of the microbe to the host tissue. After adherence has occurred, induction of an inflammatory response, release of bacterial toxins, or invasion of host tissue can follow.

Competition between indigenous microflora and new invaders

Where an indigenous microflora has established itself in the host, a competition would exist for the available adherence sites between these microorganisms and any newcomers to the area. A greater avidity for these sites by the indigenous microflora would be advantageous to the host to help prevent the establishment of a pathogen. If the pathogen is not successful in competing for a space in the host, its colonization would be impaired.

Savage, in his review (86), cites several examples where the indigenous microflora of the mammalian mucosal epithelium interferes with invading

pathogens. One example includes the indigenous microflora, lactobacilli and yeast, of the mouse stomach. As long as the lactobacilli remain and are not displaced with agents such as penicillin, other microbes seem unable to establish themselves in this area (85,86).

If the invading organism possesses a strong adherence mechanism, it can become established in the host. For example, the pathogen N. gonorrhoea, can adhere more strongly to human vaginal cells than can Lactobacillus acidophilus and other indigenous microorganisms (71,80). Pathogenic Candida albicans, is shown to adhere to, and colonize vaginal cells to a much greater degree than nonpathogenic species (57).

Forces involved in bacterial-cell surface interactions

Presently there are two schools of thought regarding the forces that mediate attachment of bacteria to a solid surface. One school suggests that the majority of the binding interactions involve electrostatic forces such as those observed when bacteria bind to glass (72), or to hydroxyapatite crystals (43). However, increasing evidence tends to support the second school, which feels that a more specific bonding occurs between bacteria and host tissue. These bonds have been likened to those involved in antigen-antibody or lectin-sugar interactions (11,12,22,42,43).

Before any bonds can be formed between a bacterial, and another cell surface, the attractive forces between them must be sufficiently strong to overcome the repulsive electrical forces created by the juxtaposition of the two negatively charged surfaces (9,11,100). Attractive forces may include van der Waals' forces where a fluctuation in charge or dipole movement could attract and draw the two surfaces together. Alternatively, suitable alignment of the two surfaces could result in the formation of

hydrogen or ionic bonds. Hydrophobic interactions between bacterial and eukaryotic cells have been suggested as a major attractive force (10,11, 12,100). Experimental results indicate that the lipid portion of lipoteichoic acids found on streptococcal cells, interacts with hydrophobic areas of eukaryotic cell membranes (10,12); this binding reaction is inhibited by gangliosides or albumen (10,12,100). Inhibition may result when the lipoteichoic acids form an interaction with the lipid moiety of gangliosides or with areas of the albumen molecule known to have a high affinity for fatty acid molecules (10,100).

Cell surface components involved in adherence

Adhering bacteria are thought to possess areas on their cell surface which are accessible and in an arrangement that facilitates the formation of bonds with complementary molecules on eukaryotic cell surfaces. Investigators studying microbial adherence are attempting to identify these cell surface components. The adhesive molecules on the bacterial cell have been termed ligands (26), or adhesins (12), and those on animal cells have been called receptors (11,12).

Experimental procedures used to determine the character of adhesins and receptors include:

- (i) Inhibiting the binding reaction with "haptens" or analogues of the adhesin, or with antibodies directed against antigens present in the adhesin.
- (ii) Modifying or destroying the adhesin with specific physical, enzymatic, or chemical treatments.
- (iii) Isolating and purifying the adhesin or receptor.
- (iv) Characterizing mutants which lack the ability to mediate the binding reaction.

Eukaryotic cell receptors

Receptors on mammalian cells have been shown to include glycoproteins, glycolipids, and proteins (12,41,43). Information on the nature of the receptors is limited but there are a few examples in the literature where the components of the eukaryotic receptor site have been determined (11,12,22,74).

Ofek and Beachey (74) have proposed that D-mannose-like residues on the surface of erythrocytes and other mammalian cells serve as receptors for type I fimbriae of Enterobacteriaceae.

Freter and others have shown that V. cholerae binds to at least two different gastrointestinal receptors (36,52,53). One is located on intestinal brush border surfaces and is L-fucose and mannose sensitive. The second whose intestinal location has not yet been determined, is L-fucose resistant (36,53).

Mycoplasma pneumoniae binds to sialic acid residues on respiratory epithelial cells (20). Treatment of the epithelial cells with neuraminidase decreases attachment by fifty percent (20). Sialic acid residues on a salivary glycoprotein serve as the receptors for saliva-induced aggregation of S. sanguis (65).

Bacterial adhesins

Bacterial cell surface components implicated in mediating adherence include polysaccharides, lipopolysaccharides, lipoteichoic and teichoic acids, proteins and lipoproteins (11,12,41,42,43,91,92,100). Some have lectin-like properties and are present in pili, fimbriae, or similar cell surface protrusions (43,74,92).

The nomenclature of bacterial protrusions involved in adherence is

currently in conflict. Pili, described as short, rigid, proteinaceous appendages that cover the bacterial cell surface, are responsible for binding some bacteria to host tissue (10,17,74,77,92). Pili, have generally been reserved for structures on Gram negative organisms. These structures have also been called fimbriae (12,26,77) and, when present on Gram positive organisms, the term often used is fibrillae.

Fimbriae are found in many species of Enterobacteriaceae, and are thought responsible for attachment of the bacteria to fungal, plant, and animal cells (26,77). These cell structures have been classified into four types according to their diameter, length, adherence properties and sensitivity of adherence to inhibition by sugars. The best studied fimbriae belong to Type I. They have been purified, shown to agglutinate erythrocytes and to adhere to tissue culture cells, both reactions are inhibited by mannose (7,26,77,92).

Studies involving fifty-nine strains of Bacteroides melaninogenicus showed that only those strains possessing fimbriae-like structures manifested hemagglutinating activities (75). Virulent strains of N. gonorrhoeae possess pili which enable them to attach to a variety of mammalian cells (17,26,77,80). Piliated gonococci bind to human cells in greater numbers and at an increased rate of attachment over those variants that lack pili (80). Buchanan and Pearce, 1976, isolated and purified gonococcal pili and found that they could agglutinate human erythrocytes (17); a reaction inhibited when anti-pili antiserum was added.

Surface appendages called fibrils, form part of a fuzzy coat on the surface of S. mitis, S. salivarius and S. pyogenes (32,39,40). These structures are believed to be important in the binding of these organisms

to respiratory epithelial cells (32,39,40).

Possession of pili or fimbriae by an organism could enhance its binding to a negatively charged surface in two ways.

- (i) They help to counteract repulsive electrostatic forces (11,26,74,77,80), since piliated bacteria have been shown to possess a lower density of negative surface charge and a more hydrophobic character (26,74). Increased hydrophobicity may assist the convergence of the two surfaces and enable a receptor-ligand bond to form.
- (ii) Pili may possess ligand sites which interact directly with receptors on the eukaryotic cell surface.

In their review, Ofek and Beachey, (74),1980, reported that increased hydrophobicity of piliated organisms such as Salmonella typhimurium and E. coli, facilitated their attachment to tissue culture cells. A similar claim has been made for the attachment of gonococci to urethral epithelium (80,93). However, other investigators feel that a more specific binding between lectin-like adhesins on gonococcal pili and sugar moieties on epithelial cells is responsible (17,77).

The biochemical nature of pili and other bacterial adhesins

The chemical composition of adhesins on bacterial cell surfaces has only been determined in a few cases.

A plasmid-coded protein, the K88 antigen, has been found on the surface of fimbriae of some enteropathogenic strains of E. coli and is believed responsible for adherence of these bacteria to the intestinal wall of piglets (54,61). Since adherence is inhibited by mannose (54,61, 91), and by methyl- α -D-mannopyranoside (7), a lectin-receptor

interaction may be involved. Other enteropathogenic strains of E. coli have a heat labile antigen distinct from the K88 antigen, thought to be associated with their adherence (26,80).

Proteins are involved in other bacterial- cell surface interactions. Adhesion of M. pneumoniae to human cells is believed to be mediated by a high molecular weight protein (20). When the bacterial cells were heat, or trypsin-treated, they lost their ability to bind to trachial epithelial cells and to erythrocytes (20). Protein A, a component of S. aureus cell walls, is known to bind to the Fc region of IgG antibodies (8). Austin and Daniels (8), have presented evidence that staphylococci localize in lung tissue infected by influenza virus, by attaching to cells coated with antiviral IgG antibodies. A protein in the cell wall of S. salivarius, mediates attachment to V. alcalescens (98,99).

The attachment of S. pyogenes to epithelial cells was originally thought to be related to the M protein present on the fibrillae of these streptococci (32). However, recent evidence from the laboratory of Beachey and colleagues (10,12), indicates that the adhesin is lipoteichoic acid. The teichoic acid is linked to the fibrillae (possibly by the M protein), and the lipid binds to hydrophobic areas on the epithelial cells (12). This would explain why treatment of streptococcal cells with trypsin would destroy their ability to bind to epithelial cells.

Lipoteichoic acids bind to oral mucosal cells, human platelets, erythrocytes, and a variety of other mammalian cells (100). The binding site on the erythrocyte appears to be a protein (100). The lipoteichoic acid present in the streptococcal cell wall has been implicated as a class of a bacterial polymer that contributes to cohesion in human dental plaque (43,74,100).

Carbohydrates, present as capsules or in fibers, have been described as the adhesin in some bacterial and eukaryotic cells (22,42). Already mentioned were the mucilaginous or holdfast material produced by some marine bacteria (21,72). Costerton et al (22), examined the surface coats of adherent bacteria in natural environments and in the mammalian host and found that many had polysaccharide fibres extending from their cell surfaces. These fibers are the adhesins that bind the microorganisms to plant, animal, or other bacterial cells. Fibers of different bacteria vary in their composition and arrangement of sugars (22). Some examples where carbohydrates act as bacterial adhesins are:

- (i) Glucans, synthesized by S. mutans, which attach to the bacterial cell wall via the enzyme glucosyl transferase and bind the organism to the tooth pellicle (40,41,43).
- (ii) Polysaccharide fibers on the surface of Bacteroides fragilis which mediate binding to rat peritoneal cells, bacteria and yeast (22).
- (iii) Dextran, which is involved in the coaggregation of A. viscosus 15987 with S. sanguis or S. mutans (15).
- (iv) An unidentified carbohydrate on the surface of S. sanguis that enables it to aggregate with A. viscosus T14V (68).
- (v) Dextran, produced by streptococci is considered to be the agent mediating their adherence to normal and damaged endocardial tissue (46,87).

The existence of bacterial polysaccharide fibers has only been determined recently from studies of bacteria in natural environments, where the production of adherent fibers confers a selected advantage to the micro-

organisms. Under controlled, noncompetitive, laboratory conditions the energy necessary to produce these fibers is probably utilized for other cellular activities (22).

Adherence properties of *S. aureus*

The virulence of *S. aureus* is accredited to its production of toxins, enzymes and free coagulase. Another property, shared by the majority of pathogenic strains, is the ability to bind to and clump in the presence of fibrinogen and its degradation products (2,18,47,48,49,50). A component on the staphylococcal cell responsible for this activity is known as clumping factor, (Clf) (29). Strains of *S. aureus* that are clumping factor positive, (Clf⁺), have also been reported to bind to immobilized fibrinogen (67).

Although some controversy exists as to whether Clf confers an increased pathogenicity to the organism, several reports in the literature attest to its importance in the colonization of *S. aureus* in the mammalian host (1,3,44,45,46). Alami et al (1), classified *S. aureus* strains into groups according to their free coagulase and Clf activities and then examined each for its pathogenicity for mice. They found that both free coagulase and Clf were significant to the pathogenesis of staphylococcal infections, with Clf playing a major role in the early stages of infection.

In another study, Gorrill et al (44), reported that after intravenous injection of mice with a variety of bacteria, only *S. aureus* localized in the mouse kidney in high numbers, the other bacteria tested were rapidly removed by the host. The investigators attributed this trait of the staphylococcal cells to the presence of kidney lodgement property (KLP) (44), and suggested it was synonymous with Clf. After further

investigation, they decided that KLP and Clf were two different proteins on the staphylococcal cell wall (45). However, they did not isolate any strain of S. aureus that was KLP⁺ and Clf⁻ and the possibility remains that the factors are the same and that Clf bound to fibrinogen in the kidney.

Other investigators (55), found that mice, injected intraperitoneally with Clf⁺ strains of S. aureus, succumbed to α -toxin produced by the bacteria. The staphylococcal cells had clumped with fibrinogen in the mouse peritoneal cavity and were thence protected from phagocytosis. Strains of Clf⁻ S. aureus were not protected and were destroyed more rapidly in the mouse.

Aly et al (3), determined that S. aureus adhered more readily to nasal mucosal cells in carriers, of this microorganism, suggesting that adherence receptors are present on the cells of these individuals but not on those of noncarriers.

Another possibility may be that carriers are more prone to skin eruptions which would create a supply of fibrinogen and fibrin for adherent Clf⁺ strains of S. aureus.

Staphylococcal endocarditis

Endocardial tissue is constantly bathed with blood, thus attachment by an organism to the endocardium and valves is viewed as the initial event in the pathogenesis of bacterial endocarditis (5,26,81,87). Individuals particularly susceptible to the disease, are those with congenital or rheumatic heart disease, with intracardiac catheters, or prosthetic valves (34,35). These conditions predispose the endocardium and valves to scar tissue and thrombi. Although S. aureus will adhere to normal valves, it shows a greater affinity for damaged endocardium or thrombi (5,27,66,81),

and has been isolated with increasing frequency from cases of bacterial endocarditis (34,35). Thrombi, composed of platelets and fibrin (5,27), may act as a nidus for Clf⁺ S. aureus. After binding to fibrin and lodging in a thrombus, further envelopment with fibrin would make the staphylococcal cell difficult to eradicate (27,89). This premise has been supported by studies of staphylococcal endocarditis produced experimentally in the rabbit (34,35,38,66,89).

Fibrinogen and fibrin

Fibrinogen, a soluble protein with a molecular weight of 340,000 daltons, is found in the blood and lymph of vertebrates (13,25). It is a dimeric molecule composed of two sets of nonidentical chains; alpha (A α), beta (B β), and gamma (C γ) interconnected by disulphide bonds. (25). The polymerization of fibrinogen results in the formation of fibrin, an essential component of normal hemostasis. This event occurs when thrombin cleaves the N-terminal ends of the alpha and beta chains releasing fibrinopeptides. The remainder of the fibrinogen molecule, the fibrin monomer, polymerizes with adjacent monomers by lateral pairing and partial overlapping to form an insoluble polymer known as non-cross-linked fibrin. (25). When the hemostatic factor XIII is present, the fibrin gel is stabilized and strengthened through introduction of covalent bonds between glycine and lysine residues on adjacent monomers. The covalently stabilized, or cross-linked fibrin, can be distinguished from the non-cross-linked form by its insolubility in 5 molar urea and by its greater resistance to enzymatic dissolution.

Clumping factor

Although there have been some reports of S. aureus clumping with soluble fibrin monomers (2,49,50,63), little is known of the interaction of the organism with a matrix of non-cross-linked or cross-linked fibrin.

The cell wall component of S. aureus called Clf has not been properly characterized, nor is it known if the same substance is involved in clumping with fibrinogen and in clumping or binding with fibrin.

McNeil (69,70), investigated the nature of clumping factor on intact staphylococcal cells. Other investigators (16,29,56,84,94), have attempted to isolate "Clf" and learn more of its physical, chemical and antigenic properties. Little information has been gained from these studies and it remains to be proven if the substance they extracted from staphylococcal cells was Clf.

Several investigators isolated a substance they called Clf from the supernatant of mechanically disrupted cells (29,56). Their preparation contained a protein that was destroyed by autoclaving or by proteolytic enzymes. The protein had no demonstrable activity with fibrinogen but could absorb or neutralize antibodies found in anti-S. aureus antiserum that inhibited fibrinogen-induced clumping of staphylococcal cells. Immuno-electrophoretic analysis failed to demonstrate if the substance had antigenic properties.

Another group extracted "Clf" with phenol from whole cells or from the supernatant of crushed cells (56,84). Latex beads, coated with their extract, agglutinated in anti-S. aureus antiserum and absorbed clumping inhibiting antibodies. However, the extract failed to react visibly in agarose gels when tested against anti-S. aureus antiserum or against antiserum from rabbits immunized with the extract. An assay was developed

by one group (56) which showed that the extract could adsorb fibrinogen, however, phenol extracts from Clf⁻ S. aureus cells or from Clf⁺ cells that had been trypsin-treated or autoclaved, also demonstrated fibrinogen adsorbing activities.

Other investigators (16,94), extracted staphylococcal cells with acid and then purified the product by isoelectric focusing. The extract was used to coat erythrocytes which then clumped in fibrinogen. Little information was given of the biochemical or antigenic properties of their extract.

Except for studies of Duthie, 1955 (30), who examined the activities of some cocci with fibrinogen, little is known of the interactions of bacteria, other than S. aureus with fibrinogen or fibrin.

Results of studies reported in this thesis attempt to:

- (i) Characterize the interactions of S. aureus and fibrin.
- (ii) Identify the staphylococcal Clf.
- (iii) Develop an assay for soluble Clf.
- (iv) Identify other bacteria which interact with fibrinogen and fibrin.

II MATERIALS AND METHODS

A. Bacteria and cultural conditions

S. aureus-1, clumping factor positive (Clf^+), was isolated from the blood of a patient with infective endocarditis. S. aureus-5, a mutant obtained from the parent strain, was free coagulase positive and clumping factor negative (Clf^-). Streptococcus Group C 12388F S. aureus D25904 (free coagulase negative and Clf^+), and A. viscosus 15987, were obtained from the American Type Culture Collection. Bacteroides, Peptostreptococci, P. aeruginosa, S. sanguis, S. mitis, S. mutans, S. salivarius, Staphylococci, E. coli, Veillonella alcalescens and Fusobacterium nucleatum, were human isolates maintained in laboratory stock culture. Inocula were kept at -70°C in growth medium supplemented with 7% (vol/vol) dimethyl sulfoxide.

The staphylococci and pseudomonas were grown in a modified trypticase soy broth (MTSB) consisting of 3.4% trypticase peptone, 0.5% NaCl, 0.3% phytone peptone, and 0.25% K_2HPO_4 . Radioactively labelled cells were prepared by growing the bacteria overnight in MTSB containing 0.05 μCi of [^{14}C] glucose per ml (Radiochemical Center, Amersham, England; 295 mCi/mmol). V. alcalescens was grown in a medium containing: 0.5% trypticase, 0.3% yeast extract, 1.5% sodium lactate and 0.1% Tween 80 (vol/vol); pH was adjusted to 7.5.

The streptococci, A. viscosus, and E. coli were grown in the medium of Gibbons and Fitzgerald (40) enriched with 0.3% yeast

extract. To obtain radioactively labelled cells, the bacteria were grown in medium supplemented with $1\mu\text{Ci}$ of [^3H] thymidine per ml. (Radiochemical Center, Amersham, England, 26 mCi/mmol).

F. nucleatum and Peptostreptococci were grown in a medium of 1.7% trypticase, 0.3% yeast extract, 0.5% NaCl, and 0.25% K_2HPO_4 . Bacteroides were grown in the same medium supplemented with 5 μg of hemin per ml.

All anaerobic bacteria were grown at 37°C in an anaerobic glove box, (Coy Mfg., Ann Arbor, Michigan) containing an atmosphere of $\text{N}_2:\text{H}_2:\text{CO}_2$ (85:10:5).

Bacteria required for clumping and adherence assays were grown for 18 h, harvested by centrifugation, washed twice with 0.005M phosphate-buffered (pH 7.0) 0.14M sodium chloride solution, (PBS) and resuspended in the same buffer, unless noted otherwise.

B. Mutagenic procedures

S. aureus-1 cells were mutagenized in Tris-maleic buffer (0.05M) pH 6.0 using N-methyl-N'-nitro-N-nitrosoguanidine at a concentration of 100 $\mu\text{g}/\text{ml}$. A culture of cells in exponential phase was exposed to the mutagen for 30 min, then washed twice with MTSB and finally resuspended in a small amount of Tris-maleic buffer. This cell suspension was mixed with media and incubated at 37°C until the culture had reached an A_{660} of 0.8. Cells were harvested by centrifugation, washed twice with PBS and resuspended in PBS.

Mutants lacking the ability to clump with fibrinogen were enriched in the following manner. Fibrinogen, final concentration of 1.0 mg/ml, was added to the cell suspension and the mixture was

shaken at room temperature for 20 min. Clumped cells were allowed to settle and the supernatant, containing unclumped cells, was removed. Fresh human fibrinogen was added to the supernatant and the enrichment procedure repeated several times. An inoculum was taken from the supernatant, added to fresh media, and the culture allowed to grow at 37°C to A_{660} of 5.0. The enrichment procedure was repeated once more except that this time the nonclumping staphylococci from the supernatant were plated onto mannitol salt agar plates. Colonies were subcultured onto MTSB and were tested for their ability to bind to fibrin and clump with fibrinogen.

C. Preparation of fibrinogen

Human fibrinogen was isolated from outdated human plasma according to the method of Blombäck and Blombäck (13). The percentage of clottable protein was 96%. Purity of the preparation was confirmed by electrophoresis in SDS 10% polyacrylamide gel (PAGE). Gels were stained with Coomassie Brilliant blue (R250 Bio-Rad, Richmond, Calif.) and revealed a single high molecular weight protein. Carbohydrate analysis, (24) demonstrated mannose, galactose, and glucosamine to be the only sugars present. The sialic acid content (96) was 0.8% wt. of fibrinogen.

D. Isolation of fibrinogen chains

Fibrinogen was reduced and alkylated to obtain polypeptide chains of: alpha (α), beta (β), and gamma (γ) by the method of Shinada and Hampton (88). Briefly, lyophilized fibrinogen (300 mg) and NaCl (525 mg) were dissolved in 10 ml of deaerated guanidine

hydrochloride at 6.0M pH 8.5. The pH was immediately adjusted to 8.5 with 1.0M NaOH and the solution incubated at 40°C for 1 h. After adding dithiothreitol (38.2 mg in 0.5 ml deaerated H₂O), and adjusting the pH to 8.8, the solution was flushed with N₂ gas for 5 min. It was then tightly capped and left at room temperature for 1.5 h. Iodoacetic acid (228.7 mg) in 3 ml H₂O was added and the pH immediately adjusted to 8.3 with 2.0M NaOH. Exactly 15 min later, glacial acetic acid was added to give a final concentration of 50% vol/vol.

The S-carboxymethylated fibrinogen was applied to a G-10 Sephadex column (2.5 x 20 cm) which had been equilibrated with 50% deaerated acetic acid and wrapped in aluminum foil. The flow rate was 30 ml/h and the eluant was monitored at A₂₈₀.

The first fraction that eluted was dialyzed against distilled water and lyophilized. Approximately 150 mg of lyophilized material was dissolved in 5 mls of 8M urea containing 0.025M sodium acetate pH 4.8 and applied to a CM-cellulose column (2.5 x 15 cm), (Bio-Rad.). The starting buffer (250 ml) of 8M urea and 0.025M sodium acetate pH 4.8 was followed by a linear gradient of 1 l 0.025M sodium acetate pH 4.8 and 1 l 0.175M sodium acetate pH 5.5 in 8.0M urea. Elution was performed at room temperature and monitored for absorbance at 280 μm. Principal A₂₈₀ fractions were collected, dialyzed against distilled water and adjusted to pH 8.5 with 1.0M NH₄OH. The fractions were concentrated by ultrafiltration with a PM-10 membrane (Amicon).

E. Preparation of fibrin vials

Both cross-linked and non-cross-linked fibrin were prepared

in glass scintillation vials. Human fibrinogen, 500 μg in 350 μl PBS was added to each vial and mixed gently with 2 units of thrombin in 50 μl PBS. To prepare cross-linked fibrin, 0.0025M CaCl_2 and 0.0125M cysteine were added to the fibrinogen solution in each vial. The vials were placed at room temperature on a reciprocal shaker (80 strokes per ml) for 2 h and then left to sit at 4°C overnight. The fibrin matrix was washed twice with 20 ml of PBS and then shaken at room temperature with PBS for 20 min. Vials were drained and left to dry at 37°C for 3 h.

The presence of cross-linked or non-cross-linked fibrin was confirmed by assaying their dissolution in 8M urea and by analysis on SDS 10% PAGE (4, 97).

F. Clumping titre of bacteria

1. Tube dilution method:

Cells from an overnight culture were washed and resuspended in PBS to a standard absorbance of 4.0 at 660 nm. The cell suspension was approximately equal to 6.4×10^9 (cells) per ml as determined by microscopic count, using a Petroff-Hausser counting chamber. Human fibrinogen 1%, was serially diluted in glass tubes with PBS. An equal volume (0.1 ml) of the cell suspension was added to each tube. PBS was substituted for fibrinogen in the control tube. Tubes were shaken for 20 min in a slanted rack at room temperature. The clumping titre was the reciprocal of the concentration of fibrinogen in the last tube showing visually detectable clumping.

2. Microtitre method:

This method essentially followed that of Leavelle et al (59).

A calibrated pipette dropper was used to deliver 0.025 ml PBS to each well of a type μ microtitre plate, (Cooke Engineering Co., Alexandria, Va.). Serial 2-fold dilutions of 2.0% human fibrinogen in PBS were made with a 0.025 ml microtitre diluter. An equal amount of a bacterial suspension, A_{660} of 4.0, was added to each well and the microtitre plate placed on a reciprocal shaker for 20 min at 25°C. A control well containing cells but no fibrinogen, was always included in the assay to determine if the bacteria self-aggregated. The end point was the last well containing clumped cells.

G. Adherence of bacteria to fibrin

Radioactively labelled bacteria were added to vials containing cross-linked or non-cross-linked fibrin. Unless stated otherwise, approximately 6.4×10^8 cells (A_{660} of 1.0) in a volume of 400 μ l PBS pH 7.0 were added to each vial which was then shaken on a reciprocal shaker for 60 min at room temperature. Nonadherent bacteria were removed by washing the fibrin layer twice with 30 ml of PBS. The vials were allowed to drain for 10 min and 10 ml of scintillation fluid was added. The amount of ^{14}C or ^3H was determined in a scintillation counter. A sample of radioactively labelled cells was also counted microscopically using a Petroff-Hausser counting chamber to relate radioactive counts and bacterial numbers. Experiments were performed in triplicate and were repeated on at least two separate occasions.

H. Characterization of the binding receptors of *S. aureus*

1. Modification of whole cells

Whole cells were treated with various physical and chemical agents to determine if any treatment affected the adherence of *S. aureus* to fibrin. Unless stated otherwise, cells were suspended in PBS pH 7.0, A_{660} of 1.0.

a. Autoclaving

Whole cells were autoclaved for 20 min at 121°C.

b. Sonication

A suspension of cells was sonicated for 5 min at 4°C with a microtipped biosonic probe (Bronwill Scientific Inc., Rochester, N.Y.). Microscopic examination showed that the cells remained intact with this procedure.

c. Acetylation

Washed cells were suspended in 10 ml of 0.05M borate buffer pH 8.5 A_{660} of 1.0. Three ml of an acetic anhydride-benzene mixture (1:2 vol/vol) were added dropwise over a thirty min time period with constant stirring in an ice bath. The pH was maintained at 8-9 by adding 1N NaOH. At the end of this reaction cells were centrifuged, washed three times with PBS and resuspended in PBS. A control contained glacial acetic acid instead of acetic anhydride.

d. SDS

Staphylococcal cells were suspended in PBS containing 1% sodium dodecyl sulphate (SDS) for 1 h at 37°C. Cells were washed five times with PBS, then shaken with Bio-Beads SM-2 (Bio-Rad), to remove residual SDS.

e. NaOH, HCl

Staphylococcal cells were treated with NaOH or HCl at 0.2N (final concentration) at 60°C for 60 min. Controls in PBS without HCl or NaOH were held at 60°C for 60 min. Cells were washed five times with PBS and resuspended in the same buffer.

f. Proteases

Whole cells of S. aureus were incubated at 37°C with each of the following proteases: trypsin, α -chymotrypsin, subtilisin, and pronase, at 1 mg/ml. Cells were washed 5 times with PBS, 4°C, to remove the enzyme. To determine if any protease remained, cells were suspended in PBS, and were incubated with Azocoll (20 mg) for 2 h at 37°C in a shaking water bath (51). Insoluble material was removed by filtering and the amount of dye released, determined by assaying the supernatant, spectrophotometrically at 520 nm. Cells were judged free of protease if no dye was solubilized during the 2 h incubation.

2. Modification of cell walls

a. Trichloroacetic acid extraction

Cell walls were extracted with 5% trichloroacetic acid (TCA), for 48 h at 4°C, or 10% TCA for 90 min at 60°C, and then washed 5 times with PBS.

b. Phenol extraction

Cell walls were mixed with 90% phenol for 15 min at 37°C, and were then washed 5 times with PBS.

c. Sodium periodate treatment

Cell walls were suspended in 0.1 M acetate buffer, pH 4.5, containing 0.01M sodium periodate and incubated in the dark at room temperature for 20 h. Control cell wall suspensions were incubated in the same buffer but without sodium periodate.

Walls were washed 3 times in PBS to remove the periodate.

d. Formamide

Cell walls (1.5 ml) at A_{660} of 4.0 in H_2O , were mixed with 1 ml formamide, and were then heated in an oil bath at $150^{\circ}C$ for 15 min. Formamide was removed by washing the walls with PBS.

Other modification procedures of cell walls were performed as described for whole cells.

I. Inhibitors of the *S. aureus* fibrin binding reaction

S. aureus cells were incubated with a number of compounds for 30 min at $25^{\circ}C$ in a shaking water bath prior to adding the cells to the fibrin matrix. The cells were incubated with the fibrin for 60 min and then assayed for adherence.

J. Isolation and solubilization of cell walls

1. Isolation of walls

Cells were harvested from MTSB and were washed twice in distilled water. Approximately 9 g (wet wt) of cells, suspended in 30 ml distilled H_2O were mixed with 10 g glass beads (75-150 μ , Bio-Rad), and the cells disrupted by stirring at maximum speed for 2 h in a Mini-mill (Gifford-Wood Co.). The glass beads were removed by filtration on a coarse sintered glass filter. The whole cells and cell

walls were separated from the filtrate by centrifugation at 40,000 x g for 15 min. Whole cells were deposited as a pellet of unbroken yellow organisms overlaid with white cell walls. The upper layer of walls was removed and washed 4 times with distilled H₂O. The purity of the wall preparation was checked by phase contrast microscopy and with a Gram stain.

2. Solubilization of walls

A 5 ml suspension of cell walls (0.2 g dry wt), in PBS was digested for 2 h at 37°C with 0.75 mg lysostaphin. Hydrolysis was measured optically by following the decrease in absorbance at 660 nm. Approximately 60-80% of the dry wt of S. aureus cell walls were solubilized in this procedure. The supernatant was separated from particulate material by centrifuging 3 times at 30,000 x g for 20 min.

K. Analysis of solubilized cell wall fractions

1. Phosphorus, protein, and hexose content

Phosphorus content of cell walls and the solubilized fraction was determined by the method of Chen et al (19). Protein was determined by the method of Lowry et al (64), using bovine serum albumin (BSA) as a standard. The amount of hexoses present in cell walls and the solubilized fraction was determined by the anthrone assay (83), (glucose standard).

2. Affinity chromatography

Sepharose 4B beads were activated with cyanogen bromide essentially according to the method of Porath et al (79). Cyanogen bromide, (300 mg per ml packed beads), was added to beads suspended in H₂O,

and the pH maintained at 11.0 by addition of 6 N NaOH. When the pH stabilized (20-30 min), the Sepharose beads were washed on a coarse sintered glass filter with 30 times their volume of ice-cold H_2O .

Purified human fibrinogen (0.5 mg/ml), in 0.1M borate buffer pH 9.0 was added to an equal volume of packed wet beads. The beads and fibrinogen were rotated at 4°C for 18 h.

The fibrinogen-coupled Sepharose beads were then washed free of uncoupled fibrinogen and were suspended in 0.1M borate buffer pH 9.0. The fibrinogen beads were assayed for their ability to bind S. aureus cells by incubating with an equal volume (0.2 ml) of radioactively labelled S. aureus cells for 30 min in a shaking water bath at 37°C. After washing the beads 3 times with PBS, the number of radio-labelled cells bound to the beads was determined in a scintillation counter. If a minimum of 40% of the S. aureus cells bound to the fibrinogen beads, the remaining beads were judged suitable for affinity chromatography.

The fibrinogen-Sepharose column (1.25 x 25 cm) was equilibrated with PBS at room temperature, then a preparation of lysostaphin-solubilized cell walls was applied. The column was eluted with PBS and the eluant monitored at 280 nm. When the A_{280} readings had returned to zero, the fraction containing Clf activity was eluted with 2.0 M NaCl in H_2O . The fractions were dialyzed overnight in distilled H_2O and lyophilized.

3. Gel electrophoresis

SDS PAGE was performed, according to the method of Ames (4) and of Weber et al (97), with 10% polyacrylamide slab gels in the presence of SDS. Proteins were stained with Coomassie brilliant

blue and carbohydrates were stained with Schiff's reagent (101).

4. Immunological procedures

Two methods for the immunization of rabbits with S. aureus -1 and S. aureus -5 were followed:

a. New Zealand white rabbits were injected subcutaneously at 1 week intervals with 4.0 mg wet wt boiled cells or 8.0 mg wet wt cell walls suspended in PBS. Cell suspensions were sterilized by boiling for 1-2 h. The injections were repeated 3 times, and antisera were collected 2 weeks after the final injection.

b. Rabbits were injected intravenously every 4 days with S. aureus -1 or S. aureus -5, (boiled 15 min) according to the following protocol:

Cells at A_{660} 1.0	Day 1	0.2 ml
	Day 4	0.5 ml
	Day 8	1.0 ml
	Day 12	1.5 ml
Cells at A_{660} 2.0	Day 16	0.5 ml
	Day 20	1.0 ml

Antisera were collected 1 week after the last injection.

To obtain absorbed antiserum, a centrifuged pellet of bacterial cells was added to an equal volume of antiserum and shaken at 37°C for 2 h and then left overnight at 4°C. Cells were removed by centrifugation and the antiserum stored at -70°C.

Immunoelectrophoresis of lysostaphin-solubilized cell wall fractions

was done in 1% agarose gels (Bio-Rad, $m_r = 0.13$), with sodium barbitol buffer 0.1M pH 8.6 containing 0.6% sodium azide. A current of 10 V/cm was applied at room temperature for 4 h.

To obtain anti-fibrinogen, anti-alpha, beta and gamma antisera, rabbits were injected subcutaneously with 400 μ g of the appropriate protein in 0.5 ml PBS and 0.5 ml Freund's adjuvant (Difco). Four additional injections were made bi-weekly, with 0.5 ml of protein only. Antisera were collected 2 weeks after the last injection.

Double immunodiffusion techniques were followed (76), to determine antibody content of the antisera.

5. Soluble clumping factor assay

a. Fibrinogen-cell clumping inhibition test

Determination of fibrinogen-S. aureus cell-clumping inhibition by antibodies present in the antiserum from rabbits immunized with S. aureus Clf⁺ cells, was performed according to the method of Rotter et al (84). To insure uniformity of the staphylococcal cells used in the assay, a stock culture of cells in PBS at A_{660} of 20.0 was stored in 2 ml aliquots at -70°C . The suspension was diluted with PBS to A_{660} of 4.0 when needed for an assay.

Antiserum, in a serial two-fold dilution in PBS, was mixed with an equal volume (0.1 ml) of the staphylococcal cell suspension. Tubes were held for 2 h at room temperature and then 0.2 ml of fibrinogen (500 μ g/ml) was added. After 5 min of rapid shaking at room temperature, the tubes were immediately checked to determine if the cells had clumped. The clumping inhibiting

titre of the antiserum tested, was recorded as the highest dilution which inhibited fibrinogen-induced clumping. Controls in the assay included cells suspended in fibrinogen, PBS, and nonimmune antiserum.

b. Soluble clumping factor assay

Fractions isolated from lysostaphin-solubilized cell walls of S. aureus by affinity chromatography, were assayed for their ability to absorb or neutralize clumping-inhibiting antibodies. An equal volume of the fraction (0.1 ml) was added to serially diluted anti-S. aureus antiserum and shaken for 30 min at room temperature. S. aureus -1 cells (0.1 ml) were added and the mixture left undisturbed for 2 h at the same temperature. Human fibrinogen 1% (0.1 ml) was added, the tubes were shaken rapidly for 5 min and then assayed for clumped cells. If clumping occurred in tubes containing antiserum which previously had showed inhibition in the fibrinogen-cell clumping assay, the substance was believed to have combined with the clumping inhibiting antibodies present in the antiserum.

When particulate material such as whole cells or cell walls were assayed, they were removed from the antiserum by centrifugation before S. aureus -1 cells were added to the assay system.

L. Hemagglutination assay

Tanned red blood cells (RBC) were coated with fibrinogen according to the method of Świtalski (94) and the assay performed in microtitre plates. Twenty-five μ l of a 10% v/v RBC suspension in PBS were added to 100 μ l of solubilized cell walls or whole cells which had

been serially diluted in PBS containing 0.25% gelatin. Controls consisted of normal RBC or RBC coated with bovine albumin (0.05%). Results were read twice, once after shaking at room temperature for 10 min, and again after shaking at room temperature for 18 h.

M. Interactions of other bacteria with fibrin and fibrinogen

Other bacteria were tested for their ability to bind to fibrin and to clump in fibrinogen. Binding to fibrin and fibrinogen-induced clumping were measured as described previously with S. aureus.

N. Adsorption of fibrinogen

The following assay was used to determine if bacteria could bind to fibrinogen, but not clump in its presence. Organisms tested were washed twice in PBS and were then suspended in the same buffer to an A_{660} of 4.0. The organisms were mixed with an equal volume (0.5 ml) of 0.2% fibrinogen in a glass tube and the mixture shaken at room temperature for 40 min. Fibrinogen was replaced with PBS in the controls. The cells were examined both visually and microscopically to determine if they had clumped. If no clumping was observed, the mixture was centrifuged in an Eppendorf microfuge (Brinkman) and the cell pellet was washed twice with PBS and then resuspended in PBS. The cells were distributed into 3 tubes and diluted such that the turbidity of the cells at A_{660} was 1.0, 2.0, and 4.0. An equal volume (0.2 ml) of S. aureus of 4.0 was added to each tube and the tubes shaken at RT for 20 min and then checked for clumped cells.

Several of the bacterial strains tested autoagglutinated; hence it was not always possible to evaluate adsorption of fibrinogen and subsequent clumping of the cells by S. aureus by detection of micro-

scopic aggregation. Consequently, attachment and clumping by S. aureus was determined by examination of Gram-stained samples of the mixtures.

O. Electron microscopy

Cells of S. aureus were checked for the presence of pili by negative staining with a 2% (wt/vol) solution of phosphotungstic acid in distilled water, (adjusted to pH 7.2 with KOH). Observations were made with a Philips EM300 electron microscope.

P. Chemicals

Trypsin (twice crystallized; 12,000 U/mg), subtilisin BPN^I type III (10.4 U/mg), ganglioside type III, lysostaphin (270 U/mg), N-methyl-N'-nitrosoguanidine, bovine serum albumin, and sugars were from Sigma Chemical Co., St. Louis, Mo. Pronase (45 KU/mg) was from Calbiochem, La Jolla, Calif; α -chymotrypsin (49 U/mg) from Worthington Biochemical Corp, Freehold, New Jersey, and Freund's adjuvant was purchased from Difco.

Other biochemicals used were of reagent grade and purchased from Fisher Scientific Co., Pittsburgh.

RESULTS

A. Characterization of the *S. aureus* - fibrin binding reaction

1. Adherence of *S. aureus* to fibrin

Previous studies in our laboratory (67), had determined the parameters of fibrinogen-induced binding and clumping of *S. aureus*. Both reactions appeared to be mediated by a cell bound material, clumping factor (Clf), which was unrelated to free coagulase, a soluble substance released by *S. aureus* in plasma. Free coagulase, a para-coagulating agent, has an enzymatic action on fibrinogen and was determined to be antigenically distinct from Clf (29). The separateness of the two substances was further substantiated when isolates of *S. aureus* were obtained that were Clf- and free coagulase positive.

The present study investigated the activities of *S. aureus* with a matrix of cross-linked or non-cross-linked fibrin. An assay was developed which determined binding of ^{14}C - *S. aureus* to fibrin-coated scintillation vials. Results showed that every strain of *S. aureus* examined that was Clf+, had the ability to bind to fibrin. Isolates of *S. aureus* that were free coagulase- and Clf+, bound to fibrin and indicated that free coagulase was not involved in fibrin-binding activities.

To facilitate the characterization of Clf, a mutant, *S. aureus*-5, was obtained from the parent strain. This mutant lacked the ability to bind to fibrin and to bind to, and clump with fibrinogen, and was called Clf-. It shared with *S. aureus*-1 all other characteristics examined

such as, colonial morphology, fermentation of mannitol, production of free coagulase and hemolysis of red blood cells (RBC) (Table I). Analysis of whole cells and cell walls by SDS 10% PAGE showed that each strain possessed the same major protein bands (unpublished data).

2. Parameters of the *S. aureus*-fibrin binding reaction

a. Cell numbers versus binding to fibrin

The influence of bacterial cell numbers on the fibrin binding reaction is described in Fig. 1. The number of *S. aureus* cells that bound to 500 µg of cross-linked fibrin increased in a linear manner with the number ($5.0 \times 10^7 - 10^9$ range) of cells added. When the number of cells added exceeded 10^9 , a plateau was reached suggesting that the binding receptor sites of the cross-linked fibrin were saturated with cells. This phenomenon did not occur when non-cross-linked fibrin was incubated with the same number of cells. Receptor sites of the non-cross-linked fibrin did not appear to be saturated with cells even when cell numbers exceeded 6.0×10^9 . Linearity was observed between the number of cells added and the number that bound, however, when the inoculum size exceeded 10^9 cells, microscopic examination revealed cell:cell interactions in addition to cell:fibrin interactions. These former interactions may have produced erroneous results.

When *S. aureus*-5 was incubated with cross-linked or non-cross-linked fibrin, the same number of cells bound, regardless of the size of the inoculum added. Indications are, that this binding was of the non-specific kind and was the same as that observed when *S. aureus* bound in low numbers to glass scintillation vials.

Table I

Comparison of S. aureus-1 and S. aureus-5

Property	Organism	
	<u>S. aureus</u> -1	<u>S. aureus</u> -5
Mannitol fermentation		
a) aerobic	+	+
b) anaerobic	+	+
Production of cell-free coagulase	+	+
Yellow colonies on blood agar plates	+	±
Cream colonies on BAP	±	+
Hemolysis	+	+
Clumping with fibrinogen		
a) slide test	+	-
b) tube test	+	-
c) microtitre assay	+	-
Binding to fibrin	+	-

Figure 1. Influence of cell numbers on the S. aureus-fibrin binding reaction.

Each reaction contained:

fibrin; cross-linked or non-cross-linked, 500 μ g and ^{14}C

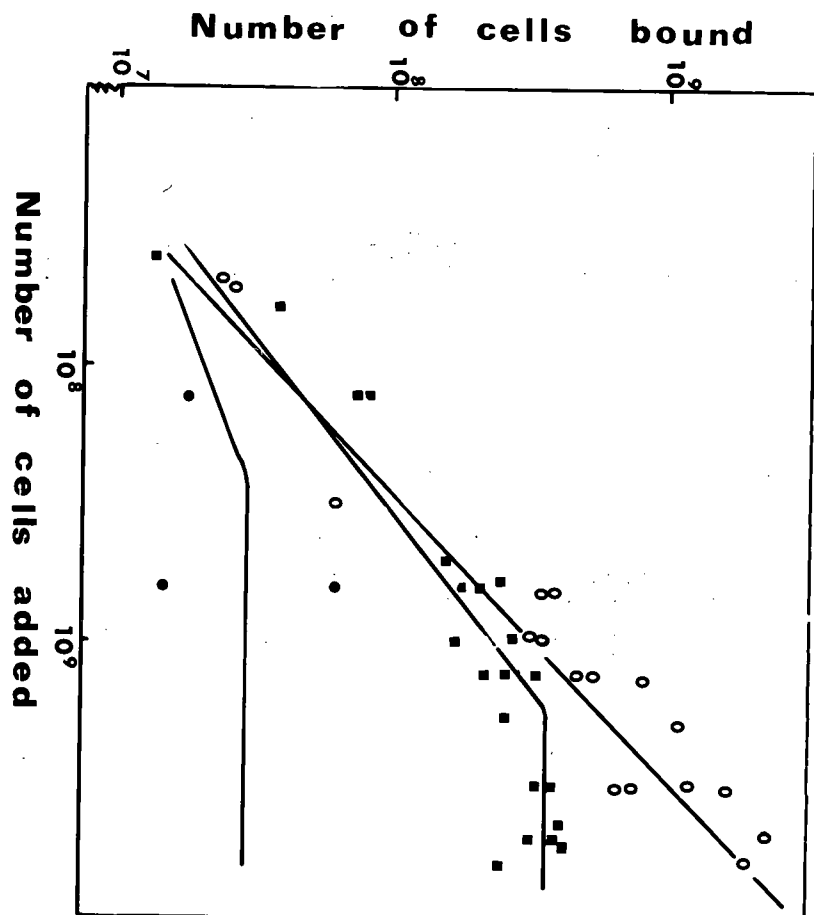
S. aureus-1 in 400 μ l PBS.

The vials were shaken gently at room temperature for 60 min. Unbound cells were removed by gently washing with PBS. Scintillation fluid was added to the washed vials and the ^{14}C measured.

0 0 0 = non-cross-linked fibrin and Clf⁺ S. aureus-1

■ ■ ■ = cross-linked fibrin and Clf⁺ S. aureus-1.

● ● ● = non-cross-linked fibrin and Clf⁻ S. aureus-5.



A standard inoculum of 6.4×10^8 cells, determined microscopically with a Petroff-Hauser counting chamber, was chosen for all subsequent experiments, unless noted otherwise. This number of cells was suspended in $400 \mu\text{l}$ of PBS, resulting in an absorbance at A_{660} of 1.0. When 6.4×10^8 cells were added to cross-linked fibrin, an average of 35% of them bound, and when added to non-cross-linked fibrin, an average of 43% bound.

b. Relationship of time of incubation to adherence

The effect of incubation time on the number of S. aureus cells that bound to fibrin is shown in Fig. 2. Adherence of cells to fibrin in both systems was very rapid; 10% of the added cells had bound to non-cross-linked fibrin after 5 min incubation and 4% to cross-linked fibrin in the same incubation time. In both systems the number of cells binding to the fibrin matrix increased with time until a plateau was reached with an incubation time of 60 min for non-cross-linked fibrin and 120 min for cross-linked fibrin. At every incubation time examined, cells consistently bound in greater numbers to non-cross-linked fibrin.

c. Effect of pH

The number of S. aureus cells binding to fibrin was investigated over a pH range of 4 to 10 and included the following buffers: 0.1M Na acetate pH 4.0, 5.0, 0.1M citrate pH 5.0, 6.0, 0.1M citrate phosphate pH 6.0, 7.0, 0.005M phosphate-buffered 0.14M sodium chloride solution (PBS) pH 7.0, 0.1M borate pH 8.0, 9.0, 0.1M boric acid-borax pH 9.0, 10.0, 0.1M boric acid - NaOH pH 10.0.

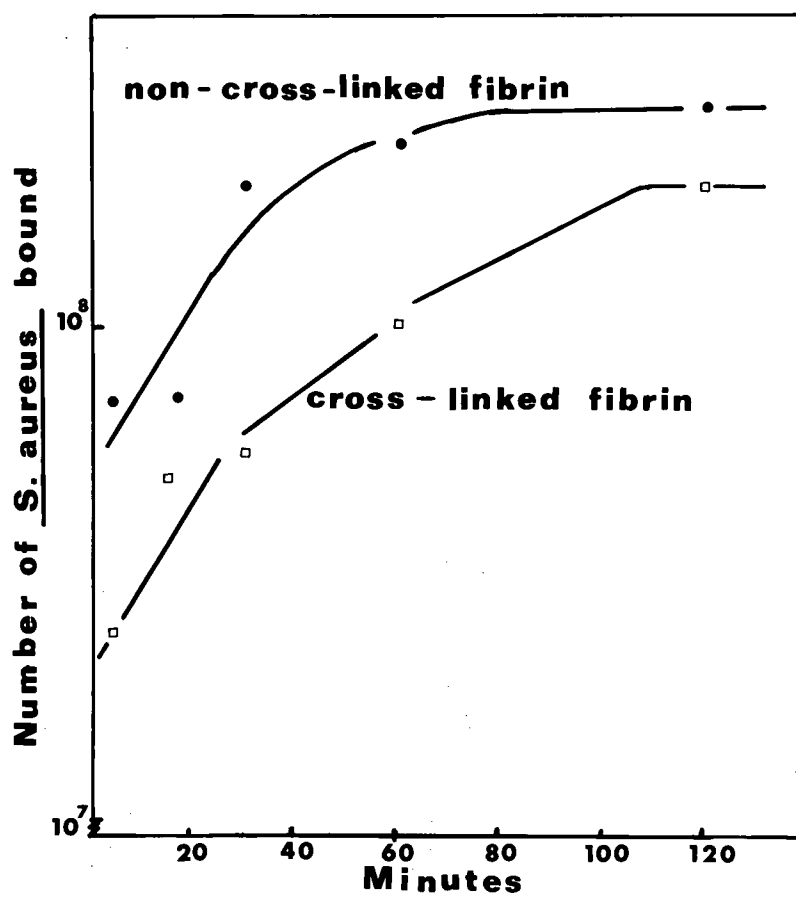
Figure 2. Effect of time on the S. aureus-fibrin binding reaction.

Each reaction mixture contained:

fibrin; cross-linked or non-cross-linked, 500 μ g, and

^{14}C S. aureus-1, 6.4×10^8 cells in 400 μ l PBS.

Vials were shaken at room temperature.



As seen in Fig. 3, binding of cells to fibrin occurred at pH 4.0, however, the high acidity of the environment caused cell:cell aggregations. The number of cells that bound to both cross-linked and non-cross-linked fibrin was lowest at pH 5.0, then increased to a maximum at pH 7.0 and remained constant to pH 10.0.

d. Temperature

The effect of temperature on the binding reaction was determined by performing the assay at 4°C, 25°C and 37°C (Fig. 4). The number of cells that bound to cross-linked fibrin was approximately the same at 4°, 25°, or 37°C. Although the number of cells that adhered to non-cross-linked fibrin was greater at 37°C than at 4°C or 25°C, results were more consistent at 25°C. For this reason, 25°C was chosen for the standard assay condition.

3. Physical and chemical treatment of S. aureus

Cells of S. aureus were treated with various physical and chemical agents to determine if any treatment modified or destroyed the cell wall component responsible for adherence of the cell to fibrin (Table II).

a. Physical treatment

Clumping factor was resistant to heating at 60°C for 1 h or 100°C for 30 min. Heating S. aureus at 100°C for 1 h or 121°C for 20 min, caused a 70 percent reduction in numbers that bound to cross-linked fibrin and a 90 percent reduction of those bound to non-cross-linked fibrin. Sonicating the cells at maximum intensity for 5 min did not alter their binding activity which suggested that the adhesin was not

Figure 3. Effect of pH on the S. aureus-fibrin binding reaction.

Each reaction mixture contained:

fibrin; cross-linked or non-cross-linked 500 μ g and
 ^{14}C - S. aureus-1, 6.4×10^8 cells in 400 μ l of the following
buffers: 0.1 M sodium acetate, pH 4.0, 5.0, 0.1 M citrate,
pH 5.0, 6.0, 0.1 M citrate phosphate pH 6.0, 7.0
0.005 M phosphate buffered, 0.14 M NaCl, pH 7.0, 8.0,
0.1 M borate pH 8.0, 9.0, 0.1 M boric acid-borax pH 9.0, 10.0
0.1 M boric acid - NaOH pH 10.0

Reaction time was 60 min at room temperature

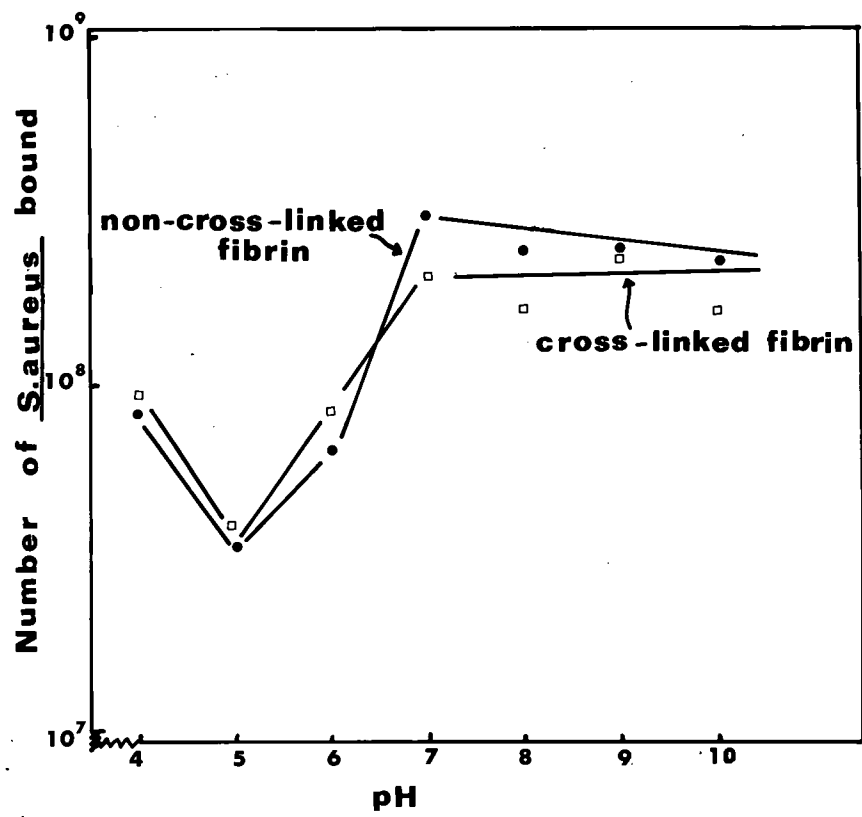


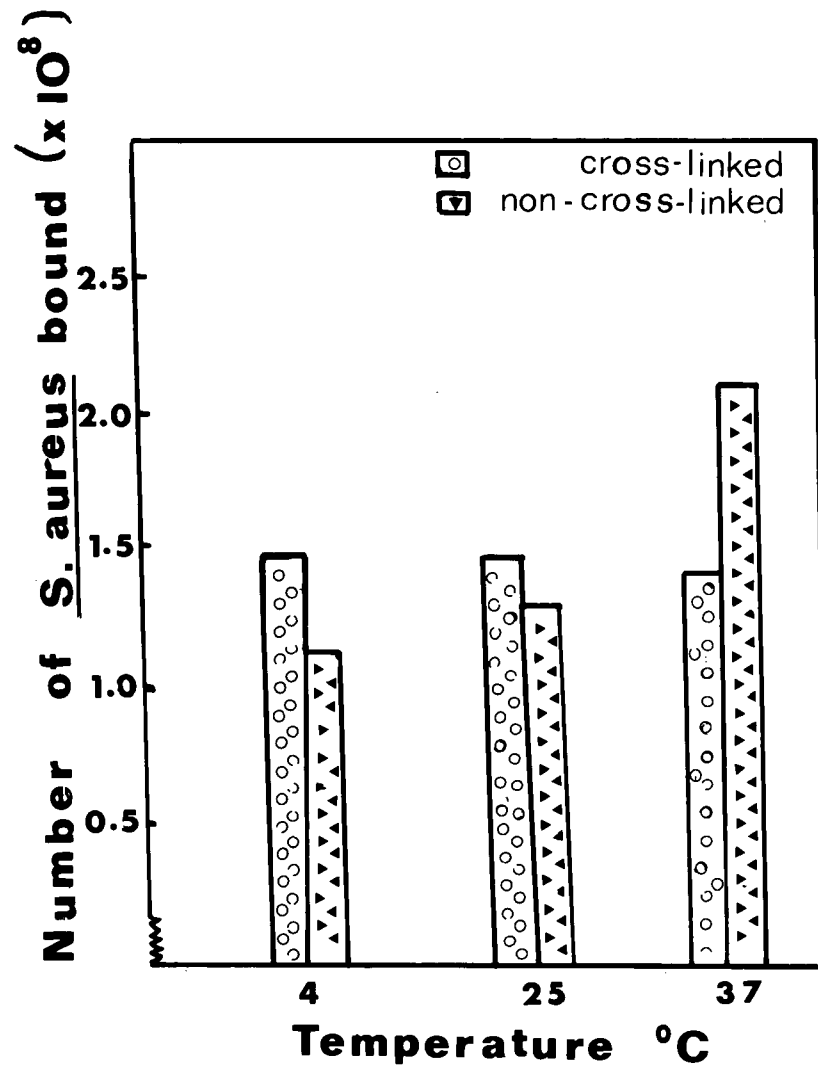
Figure 4. Effect of temperature on the S. aureus-fibrin binding reaction.

Each reaction mixture contained:

fibrin; cross-linked or non-cross-linked 500 μ g and

^{14}C - S. aureus-1, 6.4×10^8 cells in 400 μ l PBS.

Mixtures were incubated for 60 min at 4°C, 25°C, 37°C.



associated with loosely bound appendages. Electron microscopic analysis of whole cells confirmed this premise as there were no pili or similar structures observed protruding from the cell walls.

b. Chemical Treatment

As seen in Table II, extraction of the cells with SDS caused an increase in the numbers that bound to cross-linked and non-cross-linked fibrin. However, microscopic examination of the cells revealed that many had auto-agglutinated which could result in falsely elevated numbers of those bound to fibrin.

Treatment of S. aureus with 0.2N acid or 0.2N base for 1 h, caused a decrease of at least 75% in the numbers that bound to fibrin. Either the clumping factor was solubilized or destroyed by these treatments or the environment was altered in such a manner that binding interactions between the cell and fibrin molecules were no longer feasible.

Acetylation of free amino groups on S. aureus cells was performed with acetic anhydride. This procedure caused a 75% reduction in the number of cells that bound to fibrin. Acetic anhydride treatment of whole cells also eliminated their ability to bind to fibrinogen (67).

Several different hypothesis could explain why blocking free amino groups of S. aureus cells resulted in their loss of binding abilities. One possibility is that the amino groups were directly involved in the binding interaction with fibrin. Or, amino groups adjacent to the binding receptors, when blocked, posed a steric hindrance to the reaction between the cell and the fibrin molecule. Another possibility is that blockage of free amino groups on the cell surface had resulted in an increased net negative charge of the cell, creating a greater electrostatic

Table II

Effect of physical and chemical treatment on

The S. aureus-fibrin binding reaction

Treatment	Conditions	Cells bound to fibrin ⁱ	
		Cross-linked	Non-cross-linked
None		100	100
Heat	60°C, 60 min	100	100
	100°C, 10 min	111	118
	30 min	89	91
	60 min	30	10
	121°C, 20 min	27	11
Sonication	5 min	70	132
Acetylation	30 min	60	8
SDS	1%; 1h; 37°C	175	143
NaOH	0.2M; 1h; 60°C	25	14
HCl	0.2M; 1h; 60°C	11	26

ⁱExpressed as a percentage of control cells (in PBS) bound to fibrin.

barrier between itself and the fibrin molecule. Propinquity between the two surfaces was no longer favourable and the formation of bonds between them less likely to form.

4. Protease treatment

The results in Table III indicated that treatment of cells with protease, abrogated their binding abilities. Although the percentage of cells bound to fibrin was reduced by 75% after treatment for 1 h with pronase, up to 4 h of treatment with α -chymotrypsin, trypsin, and subtilisin was required to produce the same effect. If the cell wall component mediating adherence, is a protein, the results suggest that it is either resistant to protease action, or that it is protected in the cell wall environment.

5. Inhibitors of the binding reaction

An indirect analysis of the fibrin-binding factor was attempted by incubating S. aureus with a variety of compounds and then assaying the cells for binding abilities. Each compound was incubated with S. aureus for 30 min at room temperature and then the mixture was added to the fibrin layer. The substances examined were incubated with the cells to determine if any structure could act as an analogue of the binding receptor on the fibrin molecule, and bind to the adhesins on the staphylococcal cell. Subsequently, binding of the cells to the fibrin layer would be impaired. Other substances were included in the study to assess the importance of ions on the S. aureus-fibrin binding reaction. Urea was tested since previous studies showed that it prevented cells binding to fibrinogen (67).

Table III

Effect of protease treatment on the S. aureus-fibrin
binding reaction

Enzyme ⁱⁱ	Time (h)	Cell bound to fibrin ⁱ %	
		Cross-linked fibrin	non-cross linked fibrin
None		100	100
Pronase	1	27	26
Subtilisin	1	46	39
	4	33	21
α -Chymotrypsin	1	79	70
	4	25	8
trypsin	1	80	81
	4	11	7

ⁱ Results expressed as a percentage of the number of untreated cells bound to fibrin.

ⁱⁱ Concentrations of proteases were 2 mg/ml.

The results of cells, binding to the fibrin after incubation with the test material were expressed as a percentage of the number of cells binding to fibrin after preincubating in PBS. The results of the control cells in PBS were assigned the value of 100%.

Although pretreatment of S. aureus cells with 8 M urea for 1 h at 37°C had no apparent effect on the cells' ability to bind to fibrin, (unpublished data), inclusion of urea at 4.0 M in the reaction mixture of the cells and fibrin, caused a 60% decrease in the number of cells bound to fibrin (Table IV). This trend continued as the concentration of urea in the reaction mixture increased, perhaps owing to a denaturation of the fibrin molecule.

Addition of the chelating agent EDTA, had no effect on the binding reaction, suggesting that divalent cations were not involved in the interaction of S. aureus and fibrin.

None of the proteins or glycoproteins tested substantially decreased the number of cells bound to fibrin. Since gangliosides were effective in inhibiting the binding reaction, hydrophobic bonds were possibly involved in the interaction between S. aureus and the fibrin molecule. Alternatively, gangliosides may have competed with S. aureus for the same receptor sites on the fibrin molecule via a sugar-lectin type of interaction.

As is shown in Table IV all monosaccharides included in the study, caused a decrease in the percentage of cells bound to fibrin. However, the inhibition was not restricted to a specific sugar and it remains to be proven if any sugar is directly involved in the binding receptor sites.

To determine what effect the concentration of NaCl had on the binding reaction, it was added (0.14 - 2.0 N, final concentration), to the

S. aureus-fibrin mixture. As seen in Fig. 5, the number of cells that bound to fibrin, sharply decreased when the concentration of NaCl in the reaction mixture was 1 M. At 2.0 M NaCl, the percent of cells binding to cross-linked fibrin was eliminated.

To determine if NaCl (2.0 M), would cause bound cells to desorb from fibrin, the salt was added to the cell-fibrin mixture after the cells had incubated with the fibrin for 60 min. The NaCl mixture was shaken at 25°C for 30 min, then the fibrin layer rinsed 3 times with PBS and assayed for the number of cells that remained bound to the fibrin. Controls were shaken for 30 min with PBS. Results (unpublished) showed that at least 40% of the adherent cells desorbed from cross-linked fibrin and 65% desorbed from non-cross-linked fibrin. There was no desorption of cells from fibrin after shaking for 30 min with PBS.

6. Effect of antisera on the S. aureus-fibrin binding reaction

a. Antisera prepared against fibrinogen and α , β and γ chains

Antisera prepared against fibrinogen and the individual α , β and γ chains were used to determine if any antibodies present in the sera would bind to the fibrin and subsequently inhibit the binding of S. aureus. The antiserum (200 μ l) was added to the fibrin layer and incubated for 60 min at 37°C. Controls contained PBS or serum from a nonimmunized animal. The fibrin layers were washed well with PBS, and then S. aureus cells were added and assayed for adherence.

Although anti-fibrinogen, anti-alpha, anti-beta, and anti-gamma sera all inhibited binding of S. aureus to cross-linked fibrin the inhibition appeared to be nonspecific in nature, and the antibodies present in the antisera probably did not compete with S. aureus for the same receptors

Table IV
Effect of inhibitors on the S. aureus-fibrin
binding reaction

Inhibitor	Concentration	Cells bound to fibrin ⁱ %	
		cross linked	non-cross-linked
None		100	100
NaCl	1·0M	27	50
	2·0M	0	29
Urea	2·0M	117	133
	4·0M	42	42
	6·0M	25	8
	8·0M	17	4
EDTA	0·05M	120	107
BSA	2 mg/ml	87	124
Gelatin	2 mg/ml	127	85
Fetuin	2 mg/ml	93	87
Gangliosides	200µg/ml	50	74
Glucose	0·01M	71	65
Galactose	0·01M	71	65
N-acetyl glucosamine	0·01M	57	87
N-acetyl galactosamine	0·01M	64	65

ⁱ Expressed as a percentage of cells (in PBS) that bound to fibrin in the absence of any inhibiting compound.

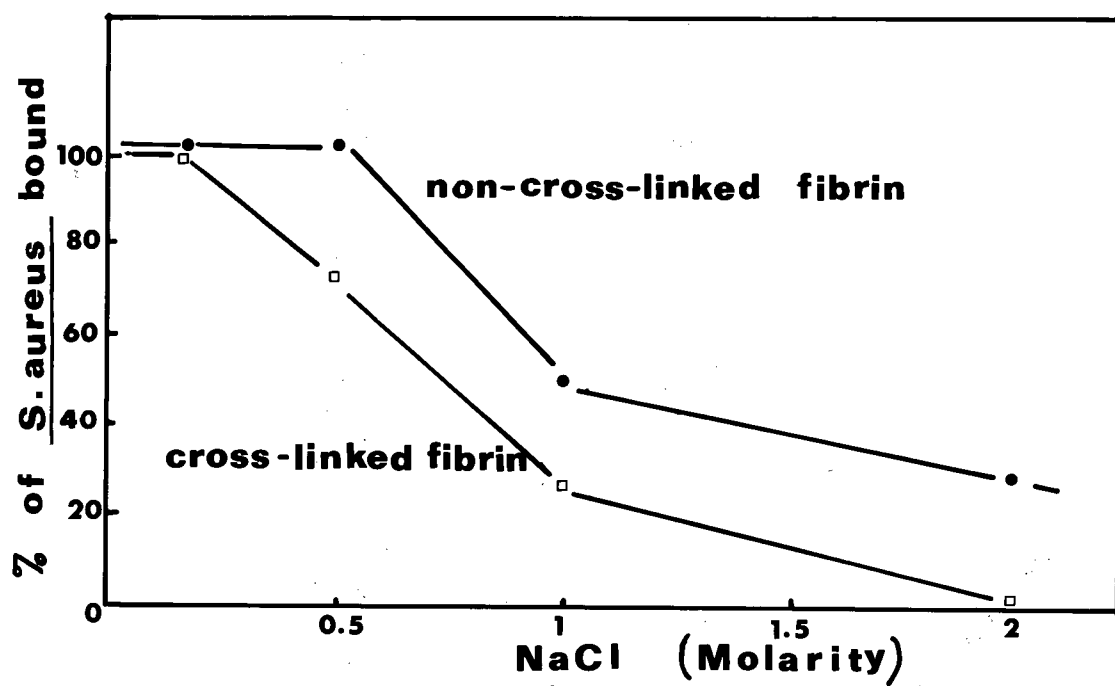
Figure 5. The effect of NaCl on the S. aureus-fibrin binding reaction.

Reaction mixtures contained:

fibrin; cross-linked or non-cross-linked, 500 μ g and

^{14}C S. aureus, 6.4×10^8 cells in 400 μ l phosphate buffer
containing 0.14 - 2.0 M NaCl.

The mixtures were incubated at room temperature for 60 min.



on the fibrin molecule but rather posed a steric hindrance to the staphylococcal cell. The antisera did not inhibit the adherence of S. aureus to non-cross-linked fibrin.

b. Antisera raised against S. aureus-1 and S. aureus-5

Prior to adding S. aureus-1 cells to fibrin, they were incubated for 30 min at 37°C with anti-S. aureus-1 antiserum, anti-S. aureus-5 antiserum or nonimmune serum. The cells were washed 3 times with PBS to remove any antibodies not bound to the cells. If the antibodies present in the antisera bound to the same receptors on the S. aureus-1 cell walls that mediated adherence to fibrin, some insight into the nature of these receptors might be gained.

As seen in Table V, incubation of cells in anti-S. aureus-1 or in anti-S. aureus-5 sera, caused a decrease in the numbers that bound to cross-linked or non-cross-linked fibrin. When anti-S. aureus-1 antiserum had been absorbed with S. aureus-1 or S. aureus-5 cells prior to incubation with S. aureus-1 cells in the assay, it retained the ability to inhibit binding of the cells to fibrin. There was no decrease in the binding activities of S. aureus-1 cells after incubation in nonimmune serum. When the cells had incubated in the antisera, they tended to clump spontaneously, and although washing the cells in PBS helped to disperse the clumps, it did not eliminate them completely. Thus, it was impossible to determine if S. aureus-fibrin binding was inhibited by specific antibodies present in the antisera or if the decrease in numbers of cells bound to fibrin was caused by a removal of clumped cells from the fibrin by the washing procedure.

Other factors such as steric hindrance by antibodies or nonspecific opsonins present in the antisera, may have also caused the same inhibiting effect.

B. Isolation and characterization of Clf

The parameters of fibrinogen-induced binding of S. aureus had previously been characterized (67), and, in this study, the S. aureus-fibrin binding reaction was examined. Was the same cell wall component involved in both of these activities, and if so, was it also synonymous with Clf? An attempt to answer these questions was made by isolating and characterizing Clf from cell walls of S. aureus.

1. Preparation of cell walls

Before Clf could be isolated from cell walls, it was first necessary to obtain cell walls which retained their clumping activities in fibrinogen. Initially, an attempt was made to break cells by ultrasonication or by homogenization in a French press. Neither of these methods was effective in disrupting the staphylococcal cell. To produce cells more susceptible to breakage, penicillin was added to the S. aureus growth medium. Unfortunately, cells grown in the presence of penicillin lost their ability to clump with fibrinogen, indicating a possible linkage between the Clf and cell wall peptidoglycan. Stirring at maximum intensity with glass beads in a mini-mill proved to be the most effective procedure for disrupting the organism. Generally, it was possible to break 50% of S. aureus cells after stirring for two h, however, there was considerable variation from one cell preparation to another. Cell walls were easily separated from unbroken cells by differential centrifugation.

Table V

Effect of antisera on the S. aureus-fibrin binding reaction

Antiserum	<u>S. aureus</u> , Clf+ bound to fibrin % ⁱ	
	Cross-linked	non-cross-linked
None (PBS)	100	100
Anti- <u>S. aureus</u> -1	45	30
Anti- <u>S. aureus</u> -5	88	38
Nonimmune serum	91	110

ⁱResults expressed as a percentage of cells incubated in PBS prior to adding them to fibrin.

2. Adherence properties of cell walls

Cell walls from the parent, S. aureus-1, retained their ability to clump in fibrinogen and, as determined microscopically, to bind to fibrin. Walls from the mutant strain S. aureus-5, did not possess either of these properties. The microtitre assay, used to determine the clumping titre of cells in fibrinogen was rapid, did not require radioactively labelled cells, and was thus chosen for studies with isolated cell walls.

3. Modification

The results of cell wall modification procedures and clumping of these cell walls in fibrinogen, correlated with similar studies done with whole cells (67), which suggested that the clumping factor in the cell wall preparation was the same as that in whole cells. Although cell walls were more sensitive to heat and protease, (Table VI), than were whole cells, all other modifications produced similar effects in both systems. In addition, cell walls were treated with phenol, TCA, sodium periodate, and formamide and were then assayed for clumping activity.

After cell walls were extracted with 5% TCA at 4°C for 48 h, no change in their clumping titre was observed, however, extraction with 10% TCA at 60°C for 90 min eliminated all clumping activities (Table VI). Control cells heated at 60°C for 90 min retained their ability to clump in fibrinogen.

Extraction of cell walls with formamide caused them to self-aggregate and thus results of their clumping in fibrinogen were difficult to interpret. Many proteins were removed from the walls after extraction with formamide as was determined by gel electrophoresis (unpublished data).

Table VI

Effect of chemical, physical and enzymatic treatment on
the fibrinogen-induced clumping of S. aureus cell wallsⁱ

Treatment	Condition	Clumping Final concentration of fibrinogen $\mu\text{g/ml}$							
		500	250	125	63	30	15	7.5	0
None		+	+	+	+	+	+	-	-
Heat	60°C, 90 min	+	+	+	+	+	+	-	-
	100°C, 15 min	±	±	-	-	-	-	-	-
Trypsin	2 mg/ml, 37°C, 2 h	-	-	-	-	-	-	-	-
Periodate	0.01M, pH 4.5, R.T. 20h	+	+	+	±	-	-	-	-
Phenol	90%, 37°C, 15 min	+	+	+	+	+	+	-	-
TCA	5%, 4°C, 48h	+	+	+	+	+	+	-	-
	10%, 60°C, 90 min	-	-	-	-	-	-	-	-

ⁱ Fibrinogen 1% was serially diluted in PBS in microtitre plates
(final vol. 0.025 ml).

S. aureus cell walls A₆₆₀ of 1.0, modified as described, were added
(0.025 ml).

The mixture was shaken for 30 min at room temperature and then the
wells were assayed for clumped cells.

Neither phenol nor sodium periodate treatment significantly altered the ability of the cell walls to clump in fibrinogen.

4. Solubilization of cell walls

After it was determined that isolated cell walls of Clf⁺ S. aureus-1 clumped in fibrinogen, an attempt was made to solubilize them and to compare the solubilized fraction with that from the Clf⁻ mutant S. aureus-5. Initially, cell walls were extracted with 10% TCA at 4°C and then treated with lysozyme, since lysozyme treatment alone produced no effect. However, this treatment failed to solubilize the walls. Therefore lysostaphin was chosen and was found to solubilize 80% of the dry wt of the cell walls after 40 min incubation at 37°C (Fig.6). Soluble material was separated from particulate matter by centrifugation. The particulate material did not clump when mixed with fibrinogen.

5. Characterization of solubilized cell walls

a. Protein, phosphorus and hexose

The protein, phosphorus and hexose content of the cell walls of S. aureus-1 and S. aureus-5 were very similar (Table VII). The total recovery of protein of the cell walls was only 52% with S. aureus-1 and 77% with S. aureus-5, but was approximately 100% of their solubilized walls. The method used to determine protein content did not account for peptides in peptidoglycan. Walls of both organisms contained similar amounts of phosphorus. Although the hexose content of the walls of both strains appeared low, the assay system used did not quantitate sugars such as those found in cell wall teichoic acid.

Figure 6. Solubilization of S. aureus cell walls with lysostaphin.

5 ml S. aureus cell walls (0.2 g dry wt.) were incubated at 37°C with 0.75 mg lysostaphin. At the designated times, samples were removed and the optical density determined.

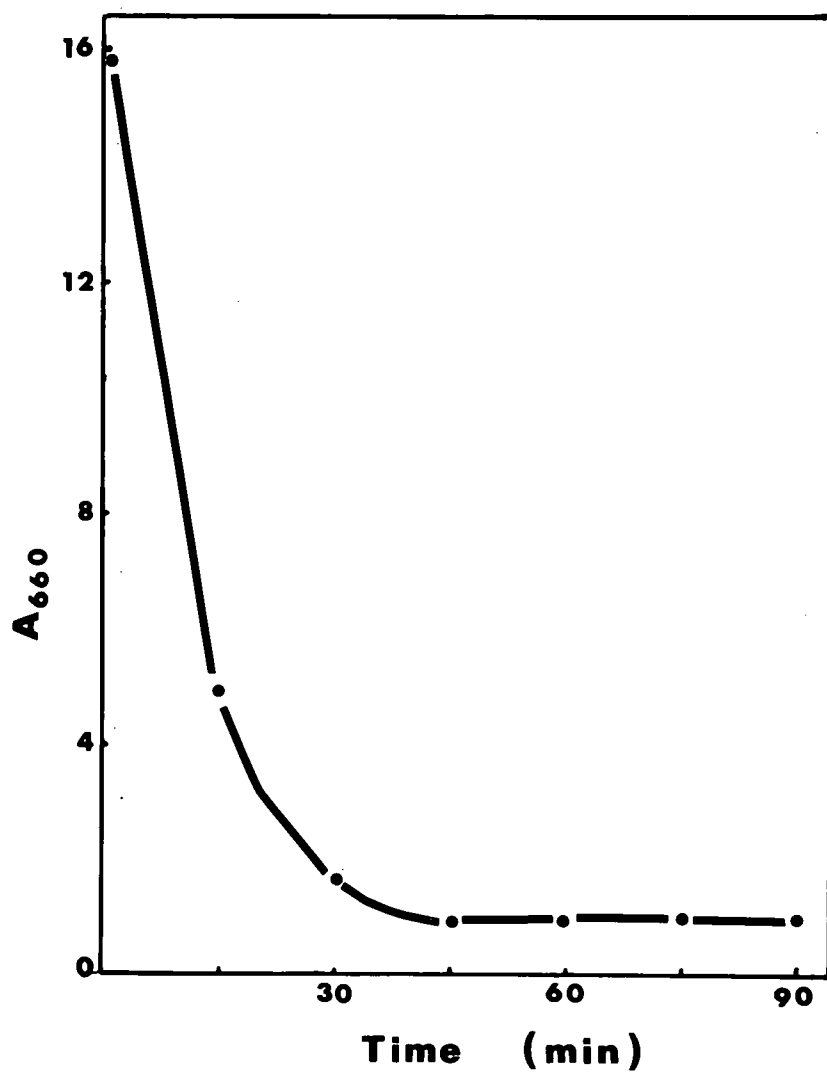


Table VII

Protein, phosphorus and hexose content of lysostaphin-solubilized cell wallsⁱ and cell walls

Organism		Dry Weight mg % ⁱⁱ		Protein mg %		Phosphorus mg %		Hexose mg %	
<u>S. aureus</u> -1	A. Cell walls	18.3	100	9.5	52	0.44	2	0.21	1
	B. Lysostaphin-solubilized	24.3	100	24.5	100	0.67	3	0.13	0.5
<hr/>									
<u>S. aureus</u> -5	A. Cell walls	16.7	100	12.9	77	0.53	3	0.31	2
	B. Lysostaphin-solubilized	11.5	100	11.3	98	0.42	4	0.03	0.3

ⁱAll values were corrected for protein, phosphorus, and hexose content of lysostaphin.

ⁱⁱValues expressed as a percent of the dry weight.

b. Immunoelectrophoresis

The antigens present in the solubilized cell walls of both S. aureus strains were examined by immunoelectrophoresis (Fig. 7 and 12). Antisera were raised against whole cells or walls of each strain. As depicted in Fig. 7 and 12, antigens, (a,b,c) were present in solubilized walls of S. aureus-1. Solubilized walls of S. aureus-5 contained only two antigens (a,b) when reacted with anti-S. aureus-5 serum (Fig. 12) or with anti-S. aureus-1 serum (Fig. 7). When anti-S. aureus-1 was absorbed with cells of S. aureus-5, the Clf⁻ strain, only one antibody could be detected which formed a precipitation arc with antigen C of solubilized S. aureus-1 cell walls (Fig. 7). The absorbed antiserum did not react with S. aureus-5 solubilized walls.

6. Isolation of Clf

To isolate Clf from solubilized cell walls of Clf⁺ strains, a selective binding of the soluble Clf onto fibrinogen-coupled sepharose beads was attempted. The fibrinogen affinity column was eluted with PBS and NaCl and monitored at A₂₈₀. As is shown in Fig. 8, a large protein peak #1 eluted in the PBS wash. A second, smaller peak #2, was eluted with 2.0 M NaCl. The fraction that eluted with 2.0 M NaCl, was present in solubilized cell walls of the Clf⁺ strain but was not present in solubilized walls of the Clf⁻ strain (Fig. 9). A summary of this method used to isolate soluble clumping factor is depicted in Fig. 10.

Figure 7. Immuno-electrophoretic analysis of solubilized cell walls of S. aureus-1 and S. aureus-5 against anti-S. aureus-1 and anti-S. aureus-1 absorbed with S. aureus-5 cells.

The antigen wells contained 25 μ l of:

- 1, 3, lysostaphin-solubilized S. aureus-1 cell walls
- 2 lysostaphin-solubilized S. aureus-5 cell walls

The antiserum troughs contained 75 μ l of:

- A. anti-S. aureus-1
- B. anti-S. aureus-1 absorbed with S. aureus-5 cells.

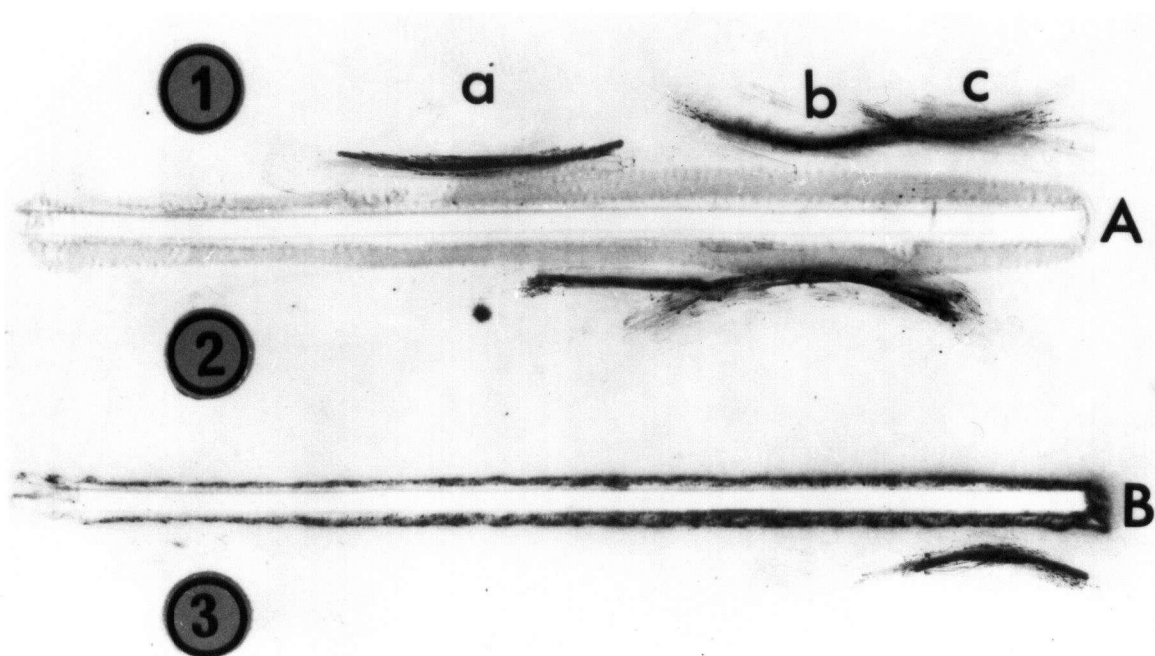


Figure 8. Fibrinogen-affinity chromatography of S. aureus-1
solubilized cell walls.

Elution profile of lysostaphin-solubilized cell walls of S. aureus-1,
measured at 280 nm.

Peak 1 eluted with PBS and peak 2, with 2 M NaCl.

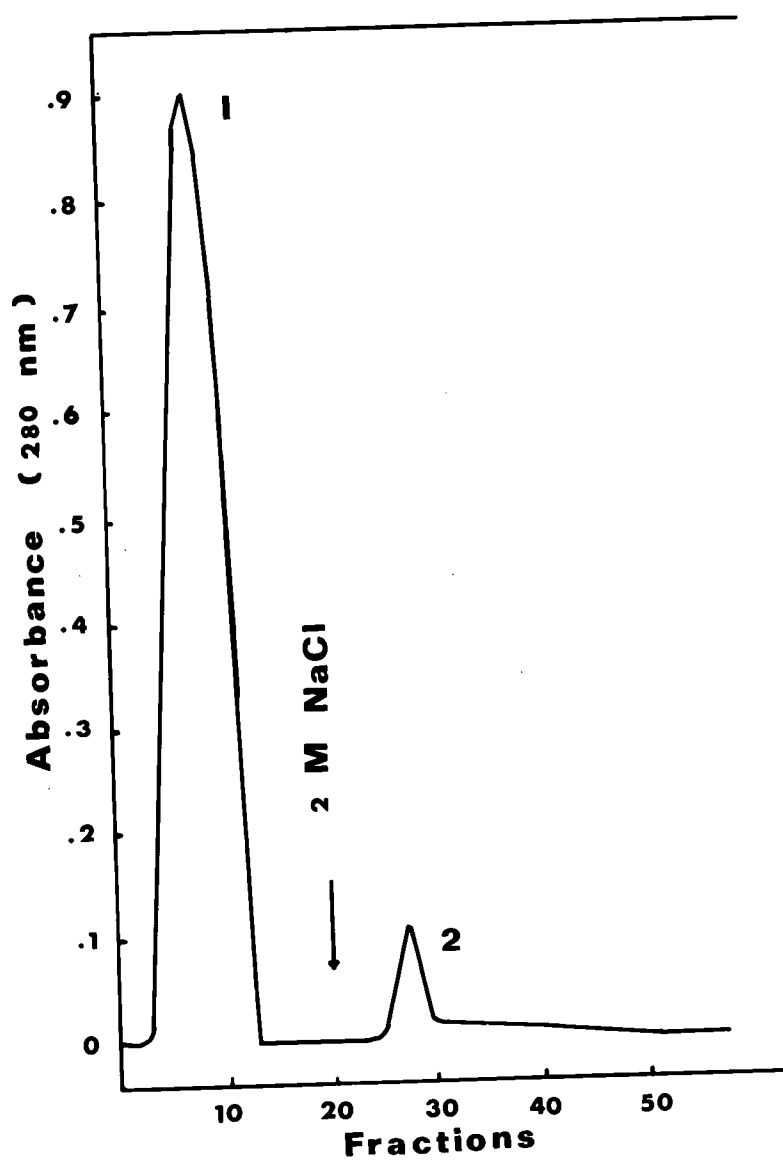


Figure 9. Fibrinogen-affinity chromatography of S. aureus-5
solubilized cell walls.

Elution profile of lysostaphin-solubilized cell walls of S. aureus-5,
measured at 280 nm.

Peak 1 eluted with PBS .

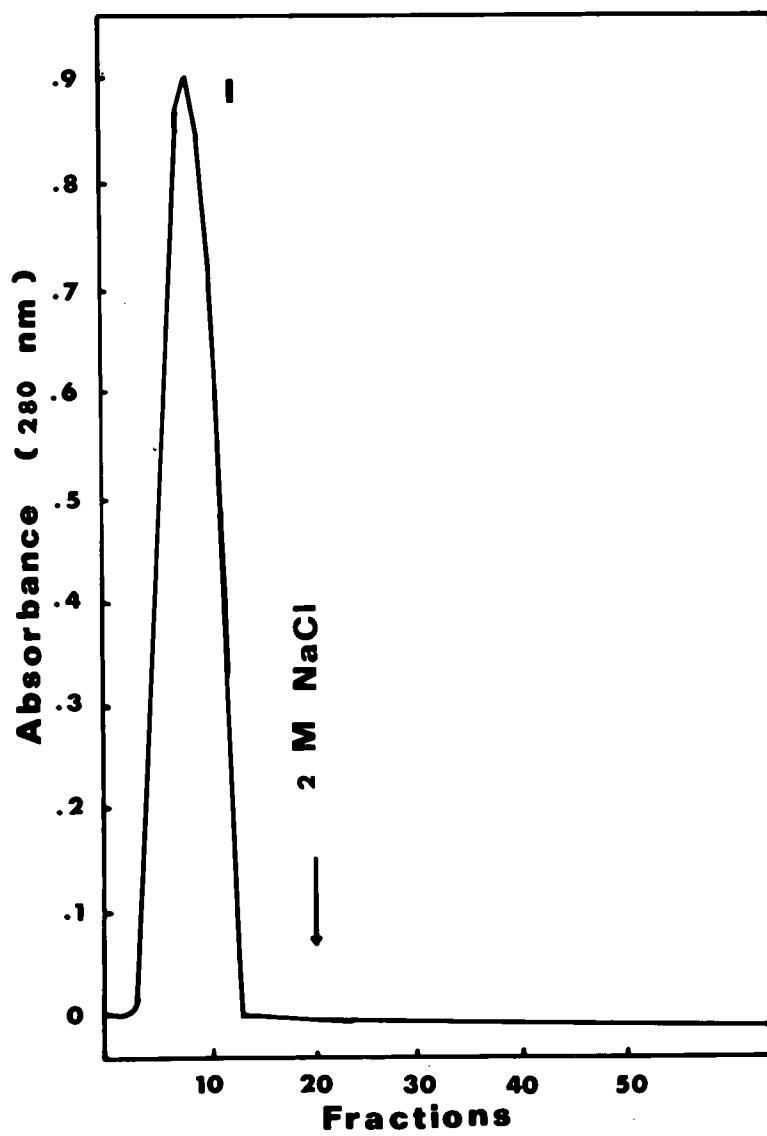
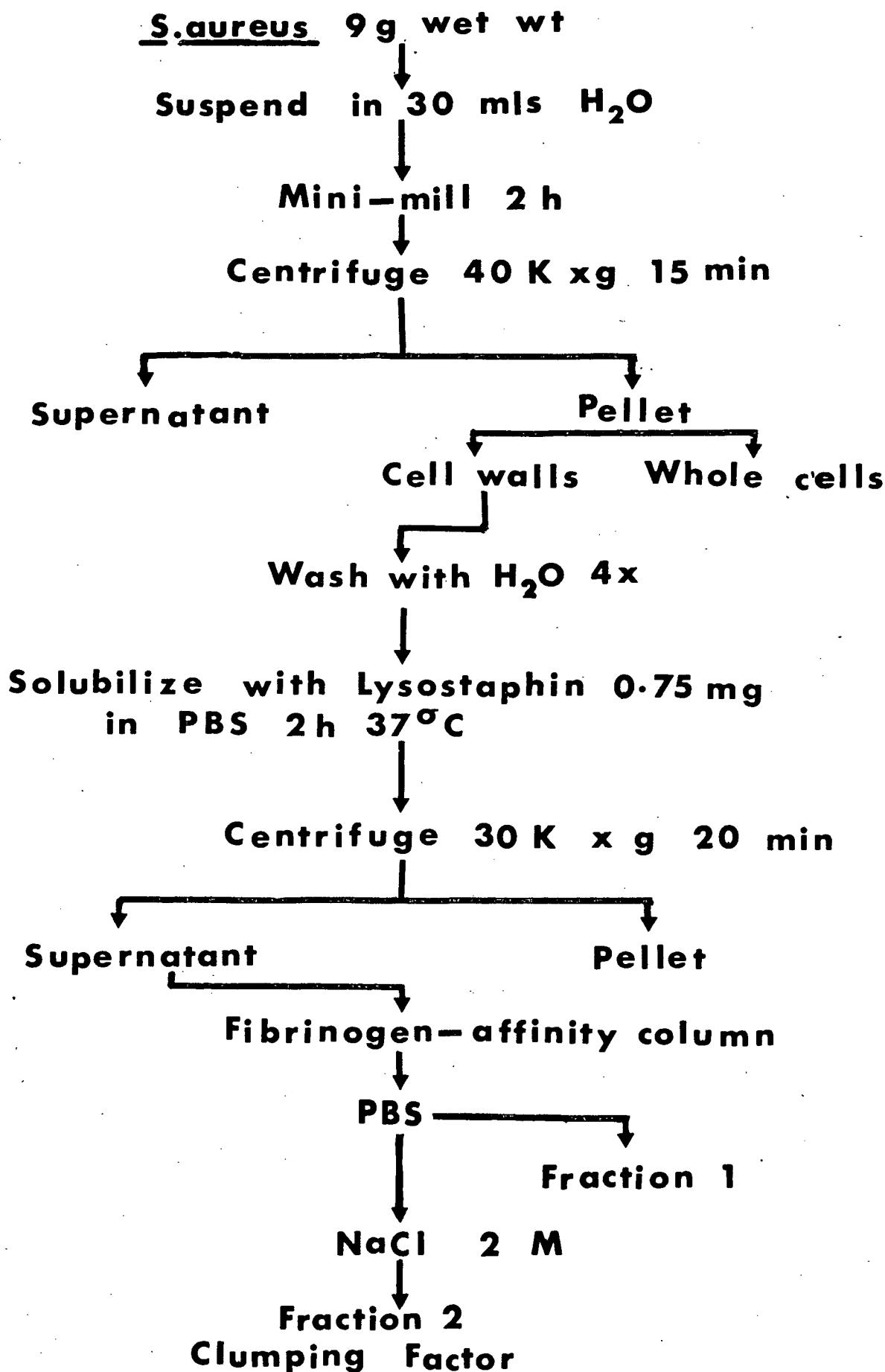


Figure 10. Protocol for the isolation of soluble clumping factor
from S. aureus.

ISOLATION of SOLUBLE CLUMPING FACTOR

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7. Characterization of Clf

a. Gel electrophoresis

The lysostaphin-solubilized walls of S. aureus-1 and S. aureus-5 and the fractions that eluted from the fibrinogen affinity column were analyzed by SDS 10% PAGE and stained with Coomassie blue and periodate-Schiff reagent Fig. 11 (a,b). A mixture of reference proteins (Bio-Rad), containing myosin (molecular weight 200,000), β -galactosidase (116,500), phosphorylase B (94,000), bovine serum albumin (68,000), and ovalbumin (43,000), were used to estimate molecular weights (unpublished data). The major proteins present in the lysostaphin-solubilized fractions of the two strains showed some differences. Walls from S. aureus-1 had six major protein bands whereas those from S. aureus-5 had nine. Of particular interest was a protein, labelled C (Fig. 11), molecular weight approximately 90,000, which was found in preparations of walls from the Clf⁺ strain, S. aureus-1, but was lacking in those of the Clf⁻ strain, S. aureus-5. This protein was the only one to stain positively with periodate-Schiff reagent. Interest in this protein increased after the analysis of the fractions, eluted from the fibrinogen affinity column, had been completed. The SDS PAGE analysis of peak #1 (Fig. 8), which had eluted with PBS from the column, revealed an identical protein profile to the preparation of S. aureus-1 solubilized walls, except that protein C was absent (lane 2, Fig. 11). Analysis of peak #2, the fraction that eluted from the column with 2M NaCl, showed that it contained a single protein, molecular weight approximately 90,000, that stained positively with periodate-Schiff (lane 3, Fig. 11). Indications were, that the (glyco)protein present in peak #2, was the same (glyco)protein C, present in S. aureus-1 solubilized walls, that had absorbed to the fibrinogen

affinity column and had desorbed with 2 M NaCl. Analysis of S. aureus-5 solubilized walls showed that protein C was absent from preparations both prior to, and after affinity chromatography. No substance could be detected to desorb from the affinity column with 2M NaCl. The unique characteristics of (glyco)protein C, namely that: (i) It was the only protein observed to stain positively with periodate-Schiff. (ii) It was present as a major constituent of the solubilized walls of the Clf⁺ strain but was lacking in those of the Clf⁻ strain. (iii) It was the only protein observed to selectively absorb to a fibrinogen affinity column, led one to believe that it might be synonymous with Clf.

Further information of the characteristics of protein C was attempted with immunoelectrophoresis and assays to determine its activities with fibrinogen and with anti-Clf antibodies.

b. Immunoelectrophoresis

The antigenic properties of the material in peak #2, were examined with antisera prepared against cell walls of Clf⁺ strains and of Clf⁻ strains. A single precipitation arc, similar to that produced with antigen C of Clf⁺ solubilized cell walls, was detected upon electrophoresis of peak #2 with Clf⁺ wall antiserum (Fig. 12). There was no detectable reaction of peak #2 with Clf⁻ wall antiserum.

c. Measurement of the activity of soluble Clf

Attempts were made to determine if the fraction isolated from Clf⁺ solubilized cell walls (peak #2), possessed fibrinogen clumping factor activity. When the soluble fraction was mixed with fibrinogen, no detectable precipitate was produced. Either an aggregate had formed but was not

Figures 11a and 11b

SDS 10% PAGE analysis of solubilized cell wall fractions of S. aureus-1 and S. aureus-5.

A 25 μ l portion of each sample was applied to an SDS, 10% polyacrylamide gel system. Proteins were stained with Coomassie brilliant blue R250, and carbohydrates with periodate Schiff reagent.

Coomassie Stained

Lane 1, lysostaphin-solubilized S. aureus-1, (Clf⁺) cell walls prior to affinity chromatography

Lane 2, lysostaphin-solubilized S. aureus-1 cell walls after affinity chromatography, peak #1, fig. 8.

Lane 3, S. aureus-1 cell wall fraction eluted from affinity column with 2 M NaCl, peak #2, fig. 8.

Lane 4, lysostaphin-solubilized cell walls of S. aureus-5 (Clf⁻) peak #1, fig. 9.

Lane 5, S. aureus-5 cell wall fraction eluted from affinity column with 2 M NaCl, fig. 9.

Periodate-Schiff.

Lane 3, S. aureus-1 cell wall fraction that eluted from affinity column with 2 M NaCl, peak #2, fig. 8.



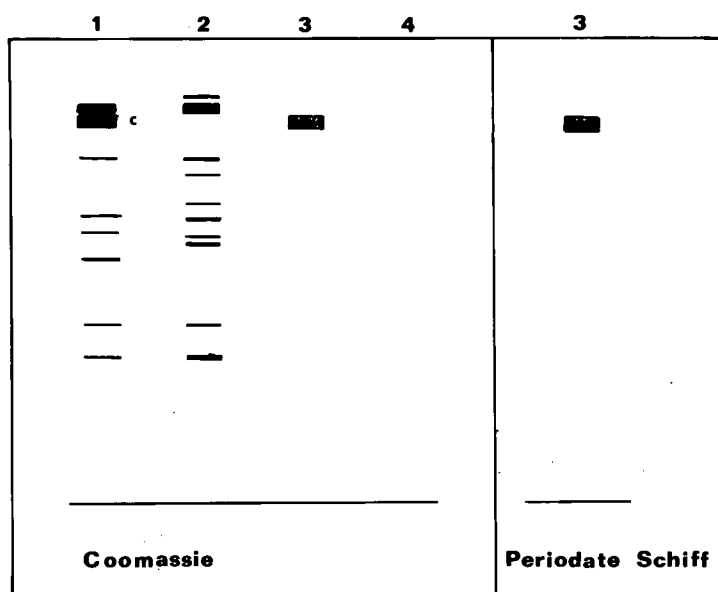


Figure 12. Immuno-electrophoretic analysis of lysostaphin-solubilized cell wall fractions of S. aureus-1 and S. aureus-5.

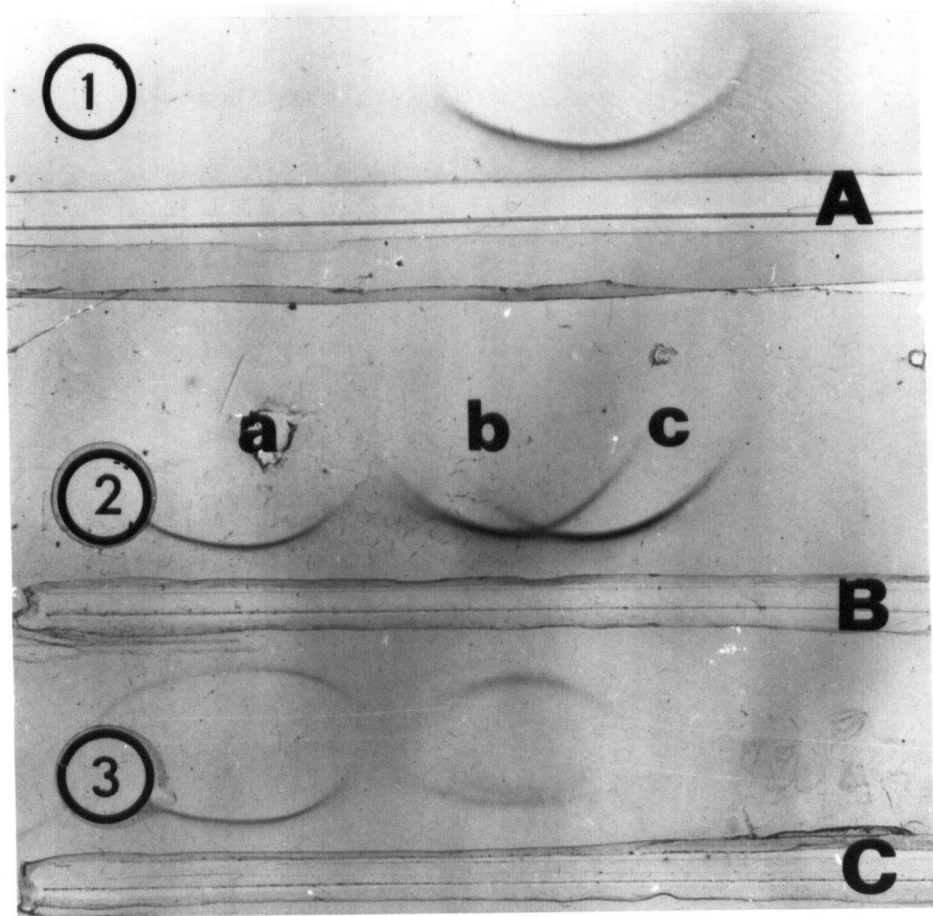
Antigen wells contained 25 μ l amounts of:

- (1) Soluble clumping factor, peak #2, fig. 8.
- (2) Lysostaphin-solubilized S. aureus-1, Clf⁺ cell walls.
- (3) Lysostaphin-solubilized S. aureus-5, Clf⁻ cell walls.

Antiserum troughs contained 75 μ l of:

A, B anti-S. aureus-1 antiserum

C anti-S. aureus-5 antiserum



visible, or, the fraction contained clumping factor in a monovalent form which was incapable of forming visible aggregates with fibrinogen.

A method developed by Świtalksi (94) to detect soluble Clf by agglutination of fibrinogen-coated tanned red blood cells (RBC), was followed. Although the RBC always agglutinated when mixed with S. aureus Clf⁺ whole cells, they did not give consistent results when mixed with soluble Clf. At times the RBC clumped spontaneously. Control RBC and RBC coated with albumin also clumped on occasion with whole cells or soluble Clf. Rotter et al (84), experienced the same difficulties with coated RBC, and thus it was concluded that the fibrinogen-coated RBC assay was not an effective assay for detecting soluble Clf.

An attempt was made to determine if solubilized Clf, isolated from S. aureus-1 cells radiolabelled with ¹⁴C, could be detected adhering to immobilized fibrinogen or to fibrin. Unfortunately, results from this assay were not conclusive.

Since methods which demonstrated fibrinogen Clf activity directly, were not effective when applied to the isolated soluble Clf, an indirect assay was developed. The assay determined if a substance could absorb specific antibodies in anti-S. aureus-1 Clf⁺, antiserum (anti-S. aureus-1) which inhibited fibrinogen-induced clumping of S. aureus Clf⁺ cells.

Antiserum prepared against S. aureus-1 cell walls, but not S. aureus-5 Clf⁻ walls (anti-S. aureus-5) contained clumping inhibiting antibodies (CIA) which inhibited the S. aureus-fibrinogen clumping reaction. Other investigators reported similar findings (29, 56, 84). As table VIII shows, S. aureus-1 cells, incubated in anti-S. aureus-1 for 2 h, did not clump when fibrinogen was added to the assay system. Clumping with fibrinogen was not inhibited when the same Clf⁺ cells were incubated in

nonimmune serum or in antiserum raised against S. aureus-5, Clf⁻ cells. Presumably, CIA were not present in anti-S. aureus-5. When anti-S. aureus-1 was absorbed with Clf⁺ cells, and then assayed, it no longer interfered with fibrinogen-induced clumping of Clf⁺ cells. It appeared that the antibodies had been absorbed by the Clf⁺ cells. The assumption is corroborated with results of anti-S. aureus-1 that had been absorbed with Clf⁺ S. aureus cells, boiled for 60 min or treated with trypsin. Both of these treatments destroyed the biological activity of Clf on whole cells or cell walls. Antiserum absorbed with these treated Clf⁺ cells retained the CIA, which suggested that boiling or treatment with trypsin also destroyed the antigenic properties of Clf on whole cells. When anti-S. aureus-1 was extracted with Clf⁻ cells, the CIA were not removed (Table VIII) and inhibited the Clf⁺ S. aureus fibrinogen clumping reaction. The preceding results indicated that there were specific anti-Clf antibodies present in anti-S. aureus-1 serum which only reacted with S. aureus-1 cells. Solubilized cell wall fractions, including soluble Clf in peak #2, were assayed in the same system to determine if any soluble material could absorb or remove the CIA from anti-S. aureus-1. The soluble test material was incubated with anti-S. aureus-1 for 30 min at room temperature, then, S. aureus Clf⁺ cells were added and, 2 h later, fibrinogen was added. After 5 min incubation, the tubes were examined for clumped cells. As seen in Table IX, soluble Clf (peak #2), reacted with CIA which thus enabled the Clf⁺ cells to clump with the added fibrinogen. Lysostaphin-solubilized cell walls of Clf⁺ S. aureus strains, also appeared to react with the antibodies in the antiserum, however, the S. aureus - fibrinogen aggregates were not as large as those observed in antiserum absorbed with soluble clumping factor. Lysostaphin-solubilized cell walls of the Clf⁻ strain

Table VIII

Influence of clumping inhibiting antibodies on
fibrinogen-induced clumping of S. aureus

Clf activityⁱ

Serum Preparation	1:8	1:16	1:32	1:64	1:128	1:256
Clf ⁺ antiserum	-	-	-	-	+	+
Clf ⁻ antiserum	+	+	+	+	+	+
Nonimmune	+	+	+	+	+	+
Clf ⁺ antiserum absorbed with Clf ⁺ cells or walls	+	+	+	+	+	+
Clf ⁺ antiserum absorbed with Clf ⁻ cells or walls	-	-	-	-	+	+
Clf ⁺ antiserum absorbed with boiled Clf ⁺ cells	-	-	-	-	+	+
Clf ⁺ antiserum absorbed with trypsin treated Clf ⁺ cells	-	-	-	-	+	+

ⁱ Serially diluted antiserum was incubated with Clf⁺ cells for 2 h at room temperature. Fibrinogen was added, the tubes shaken, and then assayed for the presence of clumped cells.

Table IX

Assay for soluble clumping factorⁱ

Sample	Dilution of Clf+ antiserum					
	1:8	1:16	1:32	1:64	1:128	1:256
PBS pH 7.0	-	-	-	-	+	+
<u>S. aureus</u> Clf+ cells ⁱⁱ or walls	+	+	+	+	+	+
<u>S. aureus</u> Clf- cells or walls	-	-	-	-	+	+
Lysostaphin-solubilized Clf+ walls	+	+	+	+	+	+
Lysostaphin-solubilized Clf- walls	-	-	-	-	+	+
Soluble clumping factor	+	+	+	+	+	+

ⁱ Sample was mixed with antiserum for 30 min prior to the addition of Clf+ cells. After 2 h incubation at room temperature, fibrinogen was added and 5 min later, the cells were assayed for clumping.

ⁱⁱ When the antiserum was incubated with whole cells or walls, they were removed by centrifugation prior to adding S. aureus-1 cells.

did not appear to absorb or remove the antibodies.

C. Reaction of other bacteria with fibrin and fibrinogen

Table X summarizes studies aimed at characterizing the fibrin-binding and fibrinogen clumping activities of a number of different bacteria.

1. Clumping in fibrinogen

The ability to clump in the presence of fibrinogen was only observed with streptococcal species of group C. Since A. viscosus and F. nucleatum auto-agglutinated, fibrinogen-induced clumping of these organisms could not be determined.

2. Binding to fibrin

Streptococcal species of groups A and C, Peptostreptococci, A. viscosus, and F. nucleatum bound to cross-linked and non-cross-linked fibrin. Several Clf⁻ isolates of S. aureus-1, and, occasionally S. aureus-5, were found to bind in low numbers to fibrin. A serum resistant strain of P. aeruginosa bound to non-cross-linked fibrin, whereas a serum sensitive strain did not bind to either form of fibrin.

3. Absorption of fibrinogen

Bacteria which did not clump in fibrinogen were assayed for their ability to absorb fibrinogen. Organisms that could absorb fibrinogen but not clump in its presence, may have monovalent or "incomplete adhesins" for fibrinogen which could bind to the molecule but not form a visible cell aggregate. If one assumes that the fibrinogen molecule is bivalent, then fibrinogen, bound to such an organism's cell surface, may have a free receptor site available for an organism such as S. aureus-1, possessing complete adhesins. The staphylococcal cells could bind to the free

Table X

Reaction of other bacteria with fibrin and fibrinogen

Bacteria	Cells bound to fibrin		Clumped in fibrinogen	Adsorbed fibrinogen
	cross-linked	% non-cross-linked		
<u>S. aureus</u> Clf ⁺	42	45	+	+
<u>S. aureus</u> Clf ⁻	-	-	-	±
<u>S. aureus</u> , free coagulase, Clf ⁺	55	47	+	+
<u>S. epidermidis</u>	-	-	-	±
<u>S. salivarius</u>	-	-	-	-
<u>S. sanguis</u>	-	-	-	±
Streptococci Group C	40	41	+	+
<u>S. mitis</u>	-	-	-	-
<u>S. mutans</u>	-	-	-	-
<u>S. pyogenes</u>	16	22	-	+
Peptostreptococci	46	40	-	+
<u>Veillonella</u> <u>alcalescens</u>	-	-	-	-
<u>A. viscosus</u>	50	40	i	+
<u>F. nucleatum</u>	17	9	i	+
<u>P. aeruginosa</u> serum resistant	-	29	i	+
serum sensitive	-	-	-	-
<u>E. coli</u>	-	-	-	-
<u>B. melaninogenicus</u> <u>asaccharolyticus</u>	-	-	-	-
<u>B. fragilis</u>	-	-	-	-

ⁱ Auto-agglutinated

fibrinogen sites and draw the other bacteria together to produce a visible cell aggregate. When S. aureus-1 was mixed with bacteria in the absence of fibrinogen, no clumping of cells occurred. As seen in Table X, several bacterial species appeared to adsorb fibrinogen and clumped when S. aureus-1 cells were added to the assay system. Every organism examined that bound to fibrin, also absorbed or bound to fibrinogen. This was also true of the reverse situation except for S. epidermidis and some Clf⁻ mutants of S. aureus which occasionally absorbed fibrinogen but did not bind to fibrin consistently, or in high numbers. An unknown cultural condition may have been responsible for the induction of formation of an incomplete receptor which enabled the organism to bind weakly on occasion with fibrin and, or fibrinogen.

Other bacteria that absorbed or bound fibrinogen, may have bound to identical fibrinogen receptors as those recognized by S. aureus-1, or they may have bound to an entirely different moiety on the fibrinogen molecule. This binding site may have acted as an analogue of a substance or a cell surface receptor to which the organism normally adheres to in its natural environment.

D. Summary

1. Comparison of the fibrin and fibrinogen binding adhesins

Results from this study have indicated that the cell wall component of S. aureus responsible for binding to fibrin, is identical to that mediating adherence to fibrinogen. The evidence for this hypothesis is summarized below:

- (i) All S. aureus strains tested that bound to fibrin, also bound to, or absorbed fibrinogen.
- (ii) Modification to whole cells which altered their

binding activity with fibrin were the same as those modifications which altered their binding to fibrinogen.

- (iii) Cell walls isolated from S. aureus could bind to both fibrin and fibrinogen in the same manner as could whole cells.
- (iv) Strains of S. aureus that bound to fibrin with a high avidity were used to isolate a solubilized cell wall component that bound selectively to immobilized fibrinogen.
- (v) Antibodies raised against S. aureus strains that bound to fibrin and fibrinogen were specific and appeared to inhibit binding of cells to fibrin and fibrinogen.

2. Relationship of Clf to fibrin and fibrinogen binding, and to fibrinogen-induced clumping.

Although every organism examined with the ability to clump in fibrinogen also bound to fibrin and immobilized fibrinogen, the reverse was not true. If the fibrinogen molecule and fibrin monomer were truly bivalent and possessed at least two identical binding receptors, and if both receptors were bound to an organism with complete, fibrinogen receptors, cell aggregates could be produced. This appears to be the situation with S. aureus-1 (Model 1, Fig. 13). Only these strains were capable of eliciting the production of specific CIA. Other bacterial strains, including S. aureus-5, may have incomplete receptors, which allows for a weak binding of cells to fibrin and fibrinogen but not formation of a cell aggregate with fibrinogen. (Model 2, Fig. 13). Another possibility is shown in Model 3, Fig. 13, where Clf receptors

are present in the cell wall but inaccessible to the majority of fibrin or fibrinogen molecules. Thus, a fibrinogen molecule may bind to a receptor on one staphylococcal cell but, owing to the fibrinogen molecule's limited size or to its configuration when bound to a masked receptor, be unable to bind to any other staphylococcal cell. The organism would then be able to absorb the fibrinogen molecule but not clump in its presence. When S. aureus-1 cells were added to S. aureus-5 cells that had absorbed fibrinogen, clumping of cells occurred. However, if the Clf possessed by S. aureus-5 was incomplete or in a masked form, its affinity for fibrinogen would conceivably be far less than that of the complete, accessible Clf possessed by S. aureus-1. These complete adhesins should be more successful in a competition for fibrinogen and may have removed the fibrinogen that had bound to S. aureus-5 cells. Thus, in the assay used to demonstrate absorption of fibrinogen by cells, it was impossible to determine if S. aureus-1 had bound to a free site on the bound, bivalent fibrinogen molecule, and caused clumping of S. aureus-5 cells, or if the fibrinogen had dissociated from the bound cells and was used to clump S. aureus-1 cells.

If Clf exists as depicted in models 2 and 3, than it would explain why S. aureus-5 cells possessing either form of the Clf would be unable to elicit the formation of specific antibody and would not have a cell wall component that could be isolated with the characteristics observed with glycoprotein C.

A final model to explain the ability of S. aureus-5 to absorb fibrinogen but not clump, is presented in Fig. 13, Model 4. This model

suggests that S. aureus-5 cells may possess complete Clf but in insufficient numbers to procure the clumping of cells.

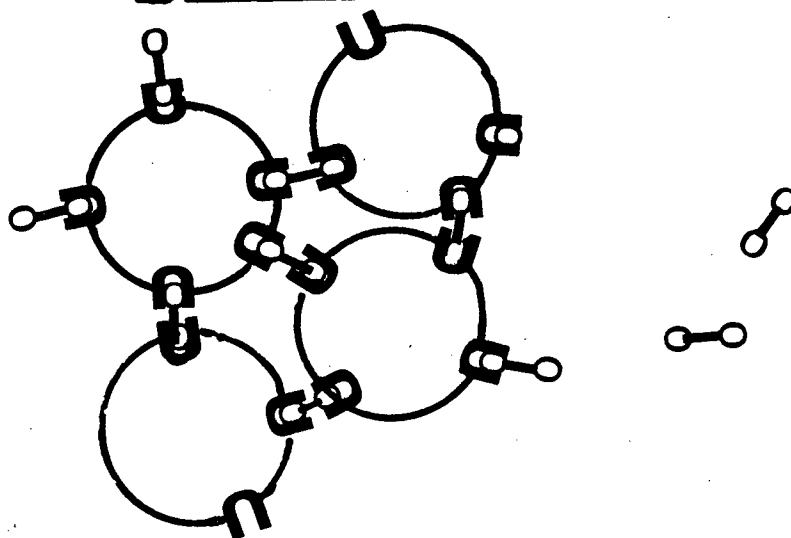
Figure 13. Models of complete, incomplete and masked clumping
factor of S. aureus.

MODELS OF FIBRINOGEN-INDUCED CLUMPING OF S. aureus

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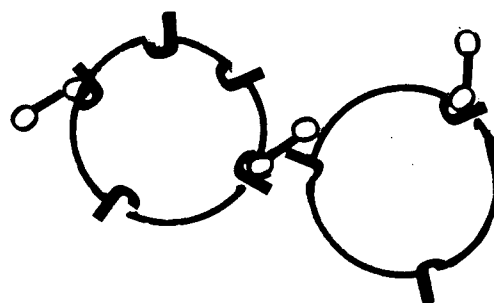
MODEL 1 S. aureus - 1 Clf +

Many,
complete
adhesins



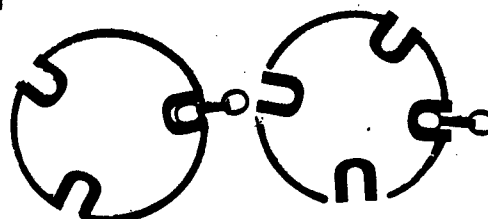
MODEL 2 S. aureus - 5 Clf -

Incomplete adhesins



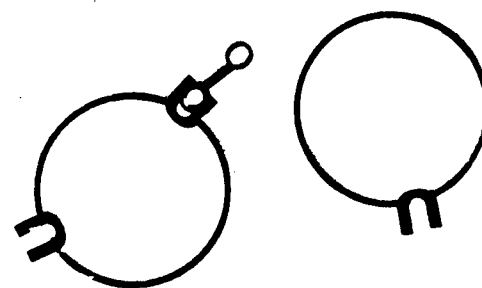
MODEL 3 S. aureus - 5 Clf -

Recessed or masked
adhesins



MODEL 4 S. aureus - 5 Clf -

Insufficient number
of adhesins



DISCUSSION

The interaction between S. aureus and fibrin has been studied and characterized, and the cell wall component mediating fibrinogen induced clumping has been identified.

The results indicate that the cell wall component, or "clumping factor" (Clf), of S. aureus which enables it to clump with fibrinogen is the same factor involved in the cell's interaction with fibrin. Strains of S. aureus that were clumping factor positive (Clf^+) clumped in fibrinogen and bound to cross-linked and non-cross-linked fibrin. Mutants, obtained from the parent Clf^+ strain lacked both properties and were called clumping factor negative (Clf^-). Previous results indicated that Clf was a separate entity of the cell, unrelated to free coagulase. This premise was further substantiated with isolates of S. aureus which produced only one of the substances.

The binding of S. aureus Clf^+ cells to fibrin was maximal at 37°C, with a pH optima of 6-8 and was independent of divalent cations. The interaction between the two surfaces occurred within minutes, and once bound to the fibrin matrix, cells were difficult to remove. Sodium chloride, 1 M inhibited binding and caused desorption of the cells from immobilized fibrin or fibrinogen. This procedure did not create any permanent damage to either constituent but may have altered their surface charge or interfered with ionic interactions between them. Adherence of S. aureus to fibrin-coated glass vials was prevented by a high

concentration of urea, this may have caused a conformational change in the Clf or fibrin molecule and thus abrogated the binding reaction.

An attempt to find a compound that could mimic or act as an analogue of the molecules involved in the binding sites of S. aureus or fibrin was inconclusive. None of the glycoproteins, proteins or glycolipids tested had an appreciable inhibitory effect on the fibrin-cell interaction. Inhibition was observed when monosaccharides were incubated with S. aureus and fibrin in the assay system; however, the inhibition was not restricted to a particular sugar. Gangliosides showed an inhibitory effect but at a concentration that suggests it was of a nonspecific nature. Gangliosides could interfere with hydrophobic bonding between the fibrin molecule and S. aureus Clf, but results of inhibition studies with albumin fail to support this premise. Albumin, known to inhibit many hydrophobic interactions (12, 100) did not have any detectable effect on the S. aureus-fibrin binding reaction.

Analysis of the interaction of fibrin and S. aureus after modification and treatment of whole cells or isolated cell walls, suggests that the adhesin or Clf is a protein. It is resistant to moderate heat (boiling 30 min but not 60 min or autoclaving) and to mild pH changes. Results of sonication procedures and electron microscopic studies suggest that Clf is not associated with surface projections such as pili or a capsule, but is rather an integral component of the cell wall, possibly attached to peptidoglycan. Methods of extraction of whole cells with lipid solvents, detergents, or phenol were unsuccessful in altering their Clf activity.

Clumping activity of whole cells was destroyed by extraction with TCA, HCl, or formamide. However, a substance with biological activity

could not be effectively removed from the cells by any of these extraction procedures. The adhesin was also inactivated by proteolytic enzymes and acetylation of amino groups. Clumping factor activity was more easily destroyed in preparations of isolated cell walls than of whole cells, which may indicate that the factor is masked in whole cells and, or, partially protected from the action of heat and protease.

Since Clf could not be physically removed or extracted from whole cells or walls, a method was devised to isolate it from a preparation of solubilized cell walls. Analysis of the solubilized cell wall preparation by SDS PAGE, revealed six Coomassie staining bands in the preparation from the Clf⁺ strain and nine bands in that of the Clf⁻ strain. Of particular interest was a protein, labelled C in Fig. 11, which had a molecular weight of approximately 90,000 daltons and that stained positively with periodic Schiff reagent. This protein was only observed in walls of Clf⁺ strains.

Immunoelectrophoretic analysis of the solubilized cell walls demonstrated an antigen, labelled C in fig. 12, present in the Clf⁺ strain and absent in the Clf⁻ strain. Only antiserum prepared against walls of the Clf⁺ strain (anti-S. aureus-1 antiserum) contained an antibody that precipitated antigen C. When anti-S. aureus-1 antiserum was absorbed with Clf⁻ cells, only the antibody precipitating antigen C could be detected in the absorbed antiserum.

The glycoprotein present in lysozyme-solubilized cell walls of the Clf⁺ strain was isolated by affinity chromatography using immobilized fibrinogen. Sodium chloride, shown previously to desorb adherent S. aureus cells from fibrinogen and fibrin, was found to be effective in eluting a fraction with clumping factor activity. This fraction was

not present when a preparation of solubilized cell walls of a Clf⁻ strain was applied to the affinity column. Analysis of the fraction by SDS 10% PAGE, revealed a single band that stained positively with Coomassie blue and with periodic Schiff and that had an identical mobility (molecular weight approximately 90,000 daltons), to that of glycoprotein C. Immunological properties of the fraction were identical to those of antigen C and a precipitin line could only be demonstrated with anti-S. aureus-1 antiserum. Immuno-electrophoretic analysis of a purified preparation of Protein A indicated that the Clf fraction was not contaminated with this cell wall component.

Material in the Clf fraction bound selectively to immobilized fibrinogen but could not be demonstrated to clump in soluble fibrinogen. An assay was developed which showed that the fraction neutralized or absorbed specific clumping inhibiting antibodies (CIA) present in anti-S. aureus-1 antiserum. No other extract or cells tested in this assay, other than those of Clf⁺ strains could absorb these antibodies and enable the S. aureus-fibrinogen clumping reaction to occur.

In summary, a substance with clumping factor activity could be isolated from cell walls of Clf⁺ S. aureus strains but not from Clf⁻ strains. It exhibited properties of a glycoprotein, molecular weight of approximately 90,000 daltons, and was antigenic. Indications are that antigen C, present in solubilized cell wall preparations of Clf⁺ strains, and the antigen in the Clf fraction are the same. The fraction formed a precipitate with an antibody present in anti-S. aureus-1 antiserum and absorbed CIA. These antibodies are probably identical. Further work is in progress to determine if the fraction will elicit production of antibodies when injected into rabbits.

Some of the characteristics of Clf such as its moderate resistance to heat and protease, its apparent antigenicity, and its property of adherence, are shared by lipoteichoic acids (LTA). Both substances are difficult to extract from cell walls and isolate in a pure form (100). However, binding reactions of lipoteichoic acids are inhibited by albumin; (10) it can be extracted from cells with cold 10% trichloroacetic acid or phenol, and is resistant to trypsin (100). These properties are not shared by the cell wall component of S. aureus exhibiting Clf activities. The possibility exists that Clf is a large peptide complex with teichoic acid as a component.

The novel method used in this study to extract Clf from a preparation of solubilized cell walls, is based on the factor's ability to selectively adhere to immobilized fibrinogen. The method appears to be more specific, efficient and doesn't involve damaging chemicals as do some of the extraction methods reported in the literature.

Although several investigators have reported isolating a substance from staphylococcal cells with Clf activity (16, 29, 56, 84, 94) proof that the substance is the Clf remains to be demonstrated.

Duthie, (1954) (29), was first in defining a method for Clf isolation. He extracted a protein, in particulate form, from an autolysate of S. aureus cells. Although the protein neutralized clumping inhibiting antibodies present in antiserum prepared against S. aureus cells, it had questionable activities with fibrinogen. The assay he used to demonstrate absorption of fibrinogen lacks specificity. Many substances examined in our laboratory by Duthie's assay system demonstrated the ability to absorb fibrinogen. These substances included non-S. aureus bacterial cells and glass.

Duthie hypothesized that a single substance with different sites for absorbing and clumping fibrinogen was present on the staphylococcal cell wall and that the clumping reaction occurred in two stages. In the first stage, an ion-independent absorption of fibrinogen occurred over a pH range of 2-11. This was followed by clumping of the cells, that required a minimum of 0.01 M sodium ions and had a pH optima of 5.5.

Two methods were employed by Kato and Omori (56) to extract a substance with Clf activity from S. aureus cells. One method used a salting out procedure to extract a substance from the supernatant of crushed cells; the other method used a phenol extraction to remove a substance from whole cells. The substances extracted by these methods could neutralize CIA from anti-S. aureus antiserum. This property of the extracts was destroyed when they were autoclaved or treated with trypsin. However, these treatments did not eliminate their ability to absorb fibrinogen. Extracts from Clf⁻ cells or from trypsin-treated Clf⁺ cells also demonstrated the ability to absorb fibrinogen in their assay system. When whole cells of Clf⁺ S. aureus are treated with trypsin or autoclaved they lose fibrinogen-absorbing and clumping activities. They explain this discrepancy by suggesting that the clumping activity of staphylococcal cell walls is dependent on two substances. One substance located within the cell is trypsin resistant, absorbs fibrinogen and can be extracted from crushed cells of Clf⁺, Clf⁻ and trypsin-treated Clf⁺ cells. The other substance, bound to the cell surface of Clf⁺ strains, is trypsin sensitive and absorbs fibrinogen and agglutinins. This substance can be removed from the cell by phenol extraction and can be separated by fifty percent acetone into a precipitate that absorbs antibodies, and a supernatant that absorbs fibrinogen.

Rotter and Kelly (84) used the method of phenol extraction to obtain a substance from staphylococcal cells which they characterized with serological tests. They coated latex beads with the extracted substance and found that anti-S. aureus Clf⁺ antiserum absorbed with Clf⁻ cells or with trypsin-treated Clf⁺ cells, agglutinated the beads. Anti-S. aureus Clf⁺ antiserum which had been absorbed with Clf⁺ cells failed to do so.

The extract did not elicit the production of detectable antibodies when injected into rabbits and did not form a precipitate with Clf⁺ anti-S. aureus antiserum.

They could not determine if the extract-latex particles clumped in fibrinogen because untreated latex beads and erythrocytes clumped spontaneously in a fibrinogen solution. This observation was corroborated in our laboratory when similar studies with untreated erythrocytes or those coated with albumin, were observed to clump in fibrinogen. The phenol extract obtained by Rotter and Kelly contained a protein which could absorb CIA but whether the protein was the Clf remains to be determined. One could speculate that their extract contained protein A, this would explain the extract's ability to bind antibody, its sensitivity to protease, but inactivity with fibrinogen. However, the inability of the extract to elicit antibody production or to precipitate with antibodies present in anti-S. aureus antiserum is not consistent with known immunogenic properties of protein A, unless, as was proposed by these investigators, their extract was not of a sufficient concentration to elicit the immune response or to precipitate with anti-S. aureus antibodies.

Attempts in our laboratory to extract Clf from whole cells or isolated cell walls with phenol were unsuccessful. After phenol treatment the cells retained their ability to clump in fibrinogen. The phenol

extract failed to show any activity with fibrinogen or fibrin and did not demonstrate protein when analyzed by SDS PAGE.

Another method reported in the literature, (16, 94) involved the extraction of a substance from S. aureus cells with formic acid, and its purification by isoelectric focusing techniques. The purified extract was reported to have Clf activities since erythrocytes coated with the extract, clumped in fibrinogen.

Indications are, that previous attempts to isolate Clf from S. aureus cells have not been wholly successful. The assays used by investigators lacked specificity, and properties of extracted Clf failed to concur with those observed for Clf whilst it was a component of intact cells. If one views Clf as a composite of two entities, with one entity capable of binding fibrinogen but both entities required for clumping of cells, an explanation for the observed discrepancies may exist. The extracts of some investigators may have contained a single component of the Clf complex and thus would have lacked the full complement of Clf properties.

Another possibility may be that their Clf extract contained protein A, another S. aureus cell wall component that shares some properties with Clf. This may explain why their extract absorbed clumping inhibiting antibodies (CIA), in anti-S. aureus serum but failed to form a precipitin line with these antibodies. Protein A is known to bind to the Fc region of IgG but would not precipitate these specific CIA.

Results from this study indicate that Clf is a single cell wall component which may however, exist in an incomplete form. The affinity for fibrinogen or fibrin by this form of Clf would not be equivalent to that of the complete adhesin, and may cause cells possessing the former to

rapidly release bound fibrinogen or to alter its configuration when bound. In either case, cross-links between separate cells, resulting in cell aggregates, would not likely occur.

Bacteria other than S. aureus were also observed to bind to fibrin or fibrinogen but not clump, indicating that they too may have possessed incomplete or masked adhesins. Or, they may have Clf in numbers too low to elicit formation of clumped cells. Alternatively, these organisms may have adhesins on their walls which merely mimic Clf activity.

Duthie (30) observed that streptococci of groups A, C and G could absorb fibrinogen but not clump in its presence. His results were corroborated in this study in addition to observations that serum resistant strains of Pseudomonas aeruginosa and Peptostreptococci also absorbed fibrinogen but did not clump.

The assumption that fibrin or fibrinogen binding is possible with bacteria that possess incomplete, masked, or inadequate numbers of Clf, or that possess adhesins analogous to Clf, suggests that assays that demonstrate binding reactions are more sensitive than those determining clumping of cells. The latter activity indicates a requirement for plentiful, complete Clf on the bacteria cell surface and may be restricted to a very few bacterial species.

The importance of Clf to the pathogenicity of S. aureus is presently under some controversy. It has been reported (55) that some virulent strains of S. aureus possess a capsule "in vivo" that mask Clf. The encapsulated strains were shown to evade phagocytosis and thus remain in the host longer than strains without capsules.

It is reasonable to assume that possession of Clf is advantageous to a staphylococcal cell when it initially encounters a surface of the

host containing fibrinogen or fibrin. The cell can attach firmly to the surface and resist removal by the host. Then, if it invades the host's tissues or cavities and no longer requires Clf, it may produce a capsule with different adherent properties, more suitable in the new environment. This adaptive mechanism could enable the bacteria to evade contact and engulfment by wandering phagocytes. In his review, Beachey (12) reports that many pathogenic bacteria with adhesins either shed or mask them with a capsule when invading host tissue.

Is a knowledge of bacterial adherence to a host's tissues purely of academic interest, or can it be used to control infections by either preventing bacterial attachment or disrupting adherent cells? Both the host and invading microbe must have their attachment sites free and accessible for a binding interaction to occur. If the host's receptor sites are blocked by purified bacterial adhesins, analogues of the adhesins, or antibodies directed against them, attachment of the bacteria could be prevented. Or, if an agent administered to the host prevents formation of the bacterial adhesin, and is harmless to the host, an infection caused by the bacteria may be avoided.

There is currently some controversy over the proposal that antibodies directed against bacterial adhesins offer more protection to a host than do antibodies directed against other bacterial surface antigens (12, 22, 92, 93).

Immunoglobulin A (SIgA), found in normal body secretions may be effective in preventing bacterial adherence, in areas such as the oral cavity. Results of several experimental studies have indicated that salivary SIgA, produced in response to cells and cell products of S. mutans, proved effective in preventing the attachment of this organism

to the tooth surface (33, 95). Other workers (14, 95) also found that SIgA diminished the numbers of S. mutans found in dental plaque, thus decreasing the incidence of caries in immunized animals. An attempt to develop an effective vaccine for the prevention of human dental caries is now in progress (33).

Several vaccines have been prepared from the antigenic fimbriae of enteropathogenic strains of E. coli. When the vaccine was administered to pregnant sows, their offspring were protected against diarrhea, after challenge with infective doses of E. coli.

Other investigators, (28) have studied the protective role of antibodies, conferred to a host, against organisms causing bacterial endocarditis. Rabbits that were immunized with S. mutans or S. sanguis and that produced a high level of specific antibody in their sera, were less likely to develop streptococcal endocarditis than animals with lower antibody titres or those not immunized with streptococci. Although these antibodies may have specifically inhibited adherence of the organism to host tissue and thus prevented the sequel of endocarditis, it cannot be discounted that agglutinated or opsonized bacteria are unquestionably more susceptible to phagocytic attack.

Analogues or substances that mimic molecules involved in the binding sites of a host or bacterium have been used in some studies to prevent bacterial adherence "in vivo". Aronson, 1979, (7) injected pathogenic strains of E. coli with methyl α -D-mannopyranoside into the bladders of mice. Methyl α -D-mannopyranoside is a known competitive inhibitor of the binding interaction between E. coli and mannose residues on host receptors. (7, 26). The presence of this compound resulted in a considerable reduction in the numbers of E. coli cells that adhered to and colonized the bladder cells.

Sublethal doses of antibiotics have been suggested as another means of preventing bacterial adherence in the host (11,12).

Sublethal doses of penicillin were found to induce S. pyogenes to secrete LTA, resulting in a loss of its ability to adhere with cellular LTA to epithelial cells (12). Our studies demonstrated that cells of S. aureus grown in a medium supplemented with a sublethal dose of penicillin could not bind to fibrin or clump in fibrinogen (unpublished observation). Thus, a better knowledge of the adherence properties of pathogenic bacteria may help prevent infections caused by them in a plant or animal host. Further examination of the isolated Clf may lead to a better understanding of its role in the staphylococcal cell and how it relates to the pathogenicity of S. aureus.

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