INFECTION AND IMMUNITY, Nov. 1988, p. 2851–2855
0019-9567/88/112851-05$02.00/0

Vol. 56, No. 11

Mediation of Adherence of Streptococci to Human Endothelial Cells by Complement S Protein (Vitronectin)

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Received 28 March 1988/Accepted 27 July 1988

The role of S protein in the adherence of group A and G streptococci to human umbilical vein endothelial cells cultivated in 96-well microdilution plates was studied by utilizing fluorescein-labeled streptococci. The assay proved suitable for quantitative determination of bacterial adherence to cultured endothelial cells for all tested strains of streptococci. Only bacterial strains with significant S protein binding but weak fibronectin binding were included in these studies. Fibronectin-mediated adherence to endothelial cells of these streptococci was less than 25% of total and could be blocked by antifibronectin immunoglobulin G. Further treatment of endothelial cell monolayers with anti-S protein immunoglobulin G at concentrations up to 1 mg per well led to an almost complete inhibition of adherence for all tested streptococcal cultures, indicating significant contribution of S protein in the streptococcus-endothelial cell interaction. Blocking of S-protein-binding sites on streptococci by preincubation with exogeneous S protein at a concentration of 10 μg per 4 × 10^7 streptococci led to about 75% reduction of S-protein-mediated adherence to endothelial cells. Trypsin pretreatment of group G streptococci and pronase pretreatment of group A and G streptococci, modifications known to destroy the bacterial binding sites of S protein, also inhibited the capacity of the streptococci for S-protein-mediated adherence to endothelial cells by 75 to 80%. These results indicate that S protein plays a mediatory role in adherence of streptococci to endothelial cells and that S-protein-specific binding sites on streptococci are involved in this interaction.

Bacterial adherence to host cells is likely to be a prerequisite for their colonization and therefore plays an important role in the initial process of infectious diseases (2, 12). Extensive studies have been carried out to understand the molecular basis for bacterial attachment to host cells (20). Pathogenic streptococci are able to bind specifically certain host plasma proteins, which may promote their adherence to host cells (4, 5, 8). One of the responsible components on the surface of human epithelial as well as endothelial cells is the adhesive glycoprotein fibronectin, which mediates adherence of certain streptococci to these cells (1, 29). Since a number of strains with only weak fibronectin-binding activities adhere equally well to most cells (P. Valentin-Weigand, G. S. Chhatwal, and H. Blobel, Am. J. Vet. Res., in press), fibronectin does not seem to be the only mediator of streptococcal adherence.

We have recently described specific interactions of another adhesive human plasma protein, complement S protein (vitronectin), with streptococci (10). Two streptococcal binding sites for S protein which were distinct from those for fibronectin were identified, one of which appeared to recognize the Arg-Gly-Asp epitope. Binding sites on group A streptococci were found to be resistant to trypsin, whereas those on group G were readily destroyed by trypsinization (10). Although S protein is different by biochemical, immunological, and structural criteria from fibronectin (13, 27), it has equivalent effects on cellular adhesion. In addition, S protein is a heparin-binding protein (25) which also expresses affinity for vessel wall-derived heparan sulfates (18), and it has recently been identified by immunofluorescence in endothelial cells (23). This prompted us to investigate the role of S protein as a mediator of streptococcal adherence to host cells, in particular to endothelial cells. In this communication, we present evidence that human S protein promotes adherence of streptococci, isolated from human infections, to cultured endothelial cells and that this interaction is mediated through the S-protein-specific binding sites on these bacteria.

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MATERIALS AND METHODS

Bacteria. A total of 15 streptococcal cultures isolated from human infections were used. Of these cultures, five each belonged to serological groups A, B, and G. Only cultures with defined binding properties for S protein (vitronectin) as previously described (5, 10) were selected. The streptococci were grown in Todd-Hewitt broth (GIBCO, Karlsruhe, Federal Republic of Germany); after 18 h at 37°C and 60 rpm they were harvested by centrifugation (20 min at 15,000 × *g*).

Culture of endothelial cells. Cultures of human umbilical vein endothelial cells were prepared by collagenase treatment as described elsewhere (14–16). Cells were identified as endothelial cells by their typical cobblestone-like morphology and positive immunofluorescence staining for von Willebrand factor. Human umbilical vein endothelial cells were grown to confluency in 96-well culture plates (Becton Dickinson, Heidelberg, Federal Republic of Germany) precoated with 0.2% gelatin (Serva, Heidelberg, Federal Republic of Germany) in Waymouth MB 752/1 culture medium (GIBCO) supplemented with 20% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine (GIBCO), and 100 μg of endothelial cell growth supplement (Sigma, Munich, Federal Republic of Germany) per ml. Human umbilical vein endothelial cells were washed twice

and incubated with serum-free medium 2 h before the adherence experiments were started. Cells of passages 1 and 2 were used for adherence assays.

S protein, fibronectin, and antisera. S protein was purified from platelet-poor human plasma as previously described (26). The purified preparation was homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17) and Western blot (immunoblot) analysis (30) and did not cross-react with antibodies against fibronectin. The functional properties of S protein were established by its ability to inhibit complement-dependent lysis, to neutralize the anticoagulant activity of heparin in the thrombin-antithrombin III reaction (25, 26), and to promote attachment of fibroblasts (24) and endothelial cells (23). Fibronectin was isolated by the method of Miekka et al. (19). Antibodies against human S protein and fibronectin were raised in rabbits, and the immunoglobulin G (IgG) fractions were isolated with a DEAE–Affi-Gel column (Bio-Rad, Munich, Federal Republic of Germany) according to the instructions of the manufacturer.

Adherence assays. A previously described fluorophotometric method (31) was modified for direct measurement of adherence of bacteria to endothelial cell monolayers in 96-well microdilution plates. Briefly, 100-μl samples of fluorescein isothiocyanate-labeled streptococci (ultrasonicated for 3 s) were added to the confluent monolayers of endothelial cells. The number of streptococci used in each assay was 4×10^7 per well as determined by optical density at 600 nm before labeling. After 1 h at 37°C under 5% CO_2 the monolayers were washed three times with Hanks balanced salt solution to remove nonadherent streptococci. The fluorescein associated with the adherent streptococci was measured in a Fluorescan II Titer-tek (Flow Laboratories, Meckenheim, Federal Republic of Germany). Each test was performed in duplicate. In control experiments, streptococcal suspensions were replaced by Hanks balanced salt solution. In parallel, streptococci were also added to gelatin-coated wells without endothelial cells to determine unspecific attachment. The degree of adherence was determined by quantitating the fluorescein label associated with the adherent streptococci by using a calibration curve constructed with serial dilutions of fluorescent streptococci. In addition, the adherence was also monitored by microscopical inspection with fluorescein isothiocyanate-labeled and unlabeled streptococci at 250 μg per well to exclude the possible effects of labeling on adherence. All adherence assays were performed on endothelial cells pretreated with antifibronectin IgG to exclude attachment through various amounts of fibronectin possibly present on these cells. This concentration of antifibronectin IgG was effective to block the fibronectin-mediated adherence of bacteria to endothelial cells as judged in preliminary experiments.

Inhibition experiments. For inhibition studies, the monolayers were incubated with increasing amounts of anti-S protein IgG (0.008 to 1 mg per well) for 30 min and washed once with Hanks balanced salt solution before the adherence assay. Normal rabbit IgG, anti-IgG, antifibrinogen, and antialbumin served as controls. For saturation of S-protein-binding sites on streptococci, 4×10^7 streptococci were incubated with increasing amounts of S protein (0.08 to 10 μg). After 40 min at room temperature the bacteria were washed with Hanks balanced salt solution and analyzed in the adherence assay. Selected strains of group B streptococci with no binding activities for S protein served as control cultures in these experiments. They were treated like group A and G streptococci.

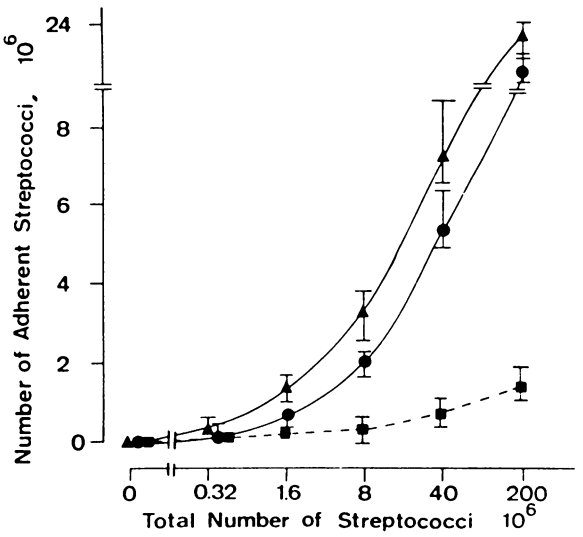

FIG. 1. Adherence of streptococci belonging to serological groups A (▲) and G (●) to cultured endothelial cells in microdilution plates as a function of number of tested organisms. The control wells (- - -) were coated with gelatin and contained no endothelial cells. The results represent means and ranges from five cultures of each serological group.

Exposure of streptococci to proteolytic enzymes. For trypsinization, 0.5-ml samples of streptococcal suspensions (4×10^8 streptococci per ml) in 0.25 M phosphate buffer (pH 7.5) were incubated with increasing concentrations (2 to 250 μg/ml) of trypsin (E. Merck AG, Darmstadt, Federal Republic of Germany) for 30 min at 37°C. The reaction was stopped by the addition of pancreatic trypsin inhibitor (Bayer, Leverkusen, Federal Republic of Germany) and subsequent washings (7). Pronase treatment was performed in 0.07 M phosphate buffer (pH 7.4) for 10 min at 40°C and subsequent washings (7). Controls were treated the same way, except that proteases were omitted. Subsequent adhesion experiments were performed as outlined above at 37°C.

RESULTS

Streptococcal adherence to endothelial cells. The fluorophotometric method was suitable for quantitative and reproducible determinations of adherence of streptococci to cultured human endothelial cells. Direct fluorescence measurements of adherent streptococci in the microdilution plates in which the cells had originally been cultured proved to be an advantage in performing the analysis. The fluorescence labeling did not alter the adherence properties of streptococci, since in control experiments with unlabeled streptococci adherence properties similar to those of fluorescence-labeled streptococci were found. Adherence of streptococci to gelatin-coated wells was less than 1% of the total, indicating a negligible unspecific attachment. All streptococcal cultures used in this study showed a concentration-dependent adherence to endothelial cell monolayers. When the logarithm of total number of streptococci was plotted against the number of adherent streptococci, the curve appeared to be sigmoidal with a linear range between 10^7 and 10^8 streptococci per well (Fig. 1). In all further adherence experiments, concentrations of streptococci in this range were used.

Influence of fibronectin in streptococcal adherence. In initial experiments, streptococcal strains with strong adherence for

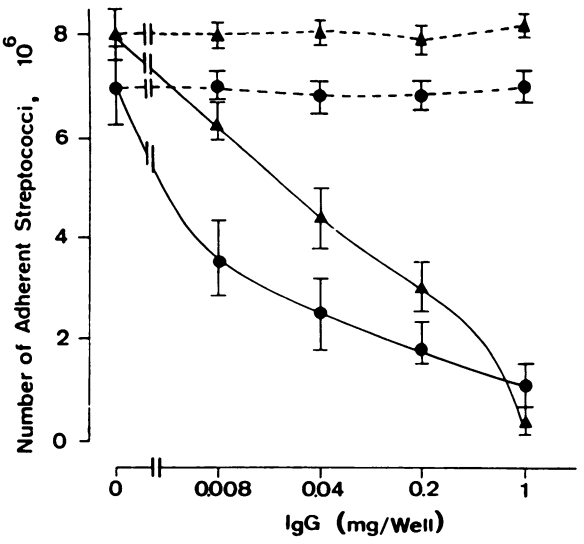

FIG. 2. Adherence of streptococci belonging to serological groups A (▲) and G (●) to cultured endothelial cells preincubated with anti-S protein IgG (——) or normal rabbit IgG (- - -). The adherence was determined in microdilution plates with 4×10^7 bacteria per well. The results represent means and ranges from five cultures of each serological group.

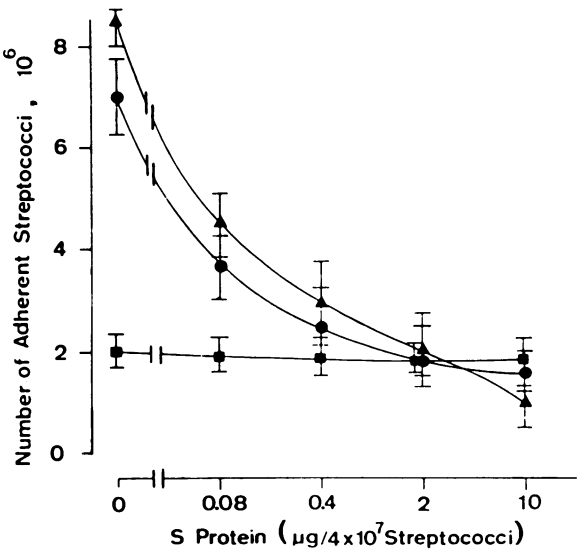

FIG. 3. Adherence of streptococci belonging to serological groups A (▲), B (■), and G (●) to cultured endothelial cells after blocking of streptococcal S-protein-binding sites with extracellular S protein. Streptococci (4×10^7) were preincubated with S protein at the indicated concentrations, washed, and used for adherence to endothelial cells in microdilution plates. The results represent means and ranges from five cultures of each serological group.

endothelial cells were selected from about 20 different streptococcus cultures belonging to serological groups A and G. Interestingly, most of these cultures showed high binding to S protein but low interaction with fibronectin. For quantitation of fibronectin-mediated adherence of these cultures, specific antibodies against fibronectin were included in the adherence assay. The inhibition caused by antifibronectin IgG was about 30 to 40% for group A streptococci and 10 to 20% for group G streptococci. Treatment of streptococci with fibronectin also led to inhibition of adherence up to 30%. The inhibition was more than 60% when the streptococci were pretreated with both fibronectin and S protein (see below). Thus, the strong adherence of these strains to endothelial cells could not be accounted for by fibronectin alone. To study the role of S protein in this respect, all further experiments were carried out with endothelial cell monolayers pretreated with antifibronectin IgG at 250 μg per well, a concentration which had been proved to cause maximal inhibition of adherence in preliminary experiments.

Role of S protein in streptococcal adherence. Pretreatment of monlayers with anti-S protein IgG led to a strong reduction of adherence for both group A and G streptococci in a dose-dependent manner. At concentrations of anti-S protein IgG as low as 8 μg per well, 30 to 50% inhibition of adherence was recognized, and at 1 mg of IgG per well the inhibition was more than 80% for both group A and G streptococci (Fig. 2). Irrelevant rabbit IgG as well as antisera against IgG, albumin, or fibrinogen used as controls did not affect streptococcal adherence.

Since the bacterial strains tested expressed significant affinity for S protein (10), exogenous S protein was used to saturate the S-protein-binding sites on the respective streptococcal strains. Pretreatment of 4×10^7 streptococci per well with native S protein decreased their adherence to endothelial cells up to 80% of the control value in a concentration-dependent manner (Fig. 3). In contrast, the adherence of group B streptococci, which have been shown to contain no binding sites to S protein (10), remained unaffected even after preincubation with 10 μg of S protein (Fig. 3).

To establish the involvement of S-protein-binding sites in the bacterium-endothelial cell interactions, proteolysis of streptococci was undertaken. Pronase treatment of streptococcal cultures, known to destroy the binding sites for S protein, resulted in a marked reduction of adherence to endothelial cell monolayers for all tested streptococcal cultures. Likewise, trypsin-sensitive binding sites on group G streptococci were also affected, such that adherence was reduced by more than 80% for these cultures. Since S-protein-binding sites on group A streptococci are relatively resistant to trypsinization, after proteolysis there was only a 5 to 10% reduction in adherence for these bacteria (Fig. 4).

DISCUSSION

Adherence of streptococci to epithelial and endothelial cell surfaces serves as a prerequisite for initiation of infections that might lead to serious diseases such as endocarditis, rheumatic fever, and glomerulonephritis (28, 32, 33). Thus, further evaluation of the molecular mechanisms of streptococcal attachment to host cells such as cells of the vessel wall is of considerable importance to understand the basis of these infectious diseases (21). The use of confluent endothelial cell monolayers was taken as a suitable model in the present study to investigate the interaction of streptococci with the vessel wall.

The adherence of streptococci to monolayers of cultured human endothelial cells was carried out by a fluorophotometric assay (31), which was modified for direct fluorescence measurements on microdilution plates. This method not only enabled quantitative and reproducible determinations of adherence but also was suitable for assaying large numbers of samples simultaneously.

Many strains of streptococci interact with host proteins either to facilitate their adherence or to prevent phagocytosis

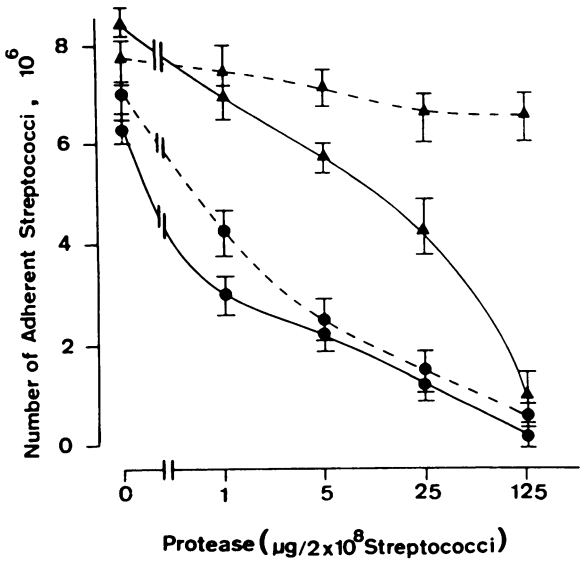

FIG. 4. Effect of proteolysis of S-protein-binding sites of streptococci on their adherence to cultured endothelial cells. Streptococci belonging to serological groups A (▲) and G (●) were treated with pronase (——) or trypsin (- - -), washed, and then used in the adherence assay. The results represent means and ranges from five cultures of each serological group.

(3, 6, 9, 32, 34). It is know from previous studies that fibronectin is present on endothelial cells and mediates adherence of certain streptococci by interactions with specific binding sites on these bacteria (29, 32). In the present study we evaluated a possible role of S protein in streptococcal adherence. Although we used selected strains with low fibronectin and high S-protein-binding activities, the endothelial cells were always pretreated with antifibronectin IgG to avoid any mediatory effect of fibronectin.

The mediatory role of cell-associated S protein in the adherence of the tested streptococci was established in a series of experiments. Blocking of cell-bound S protein with anti-S-protein antibodies led to a significant reduction of adherence of all tested streptococci, whereas various unrelated control antibodies were ineffective in this respect. S protein on the monolayers could originate either from de novo biosynthesis by endothelial cells or from the fetal calf serum used in the culture medium. Although human endothelial cells appear to be positive in immunohistochemical staining for S protein (23), the latter situation seems to be more likely since exogenous S protein may bind to heparan sulfate, which is present at the luminal surface of endothelial cells (18). Furthermore, the blocking of bacterial S-protein-binding sites by saturation with extracellular S protein resulted in a marked inhibition of adherence of group A and G streptococci but not of group B streptococci, which do not bind S protein (10), indicating a strict requirement for interaction with specific host proteins before bacterial adhesion can occur.

To further confirm that specific binding of S protein to the streptococci mediates their attachment to the endothelial cells, streptococcal binding sites were destroyed by protease treatment. In a previous study it was established that pronase affected all streptococcal binding sites for S protein, whereas trypsin was only effective on group G streptococci (10). In the present study all tested cultures gave a significantly reduced adherence to the endothelial cells after pronase treatment. Trypsinization, however, strongly reduced the adherence of group G streptococci, whereas the adherence of group A streptococci remained relatively unaffected after proteolysis. These results not only corresponded well with the findings about different S-protein-binding characteristics of both streptococcal species (10) but also demonstrated the requirement for specific interaction of bacteria with adhesive proteins before the adhesion process. The particular binding sites for S protein on these bacteria have not been identified.

The consequences of the mediatory role of S protein in bacterial adhesion to host cells such as endothelial cells remain to be clarified. On the one hand, S protein-streptococcus interaction may lead to deposition of the bacteria in various tissues such as the kidney. Interestingly, in renal tissue from patients with glomerulonephritis or arterionephrosclerosis, S protein was histochemically colocalized in immune deposits (11), indicating that it may participate in pathogenesis of renal damage caused by bacterial infection. On the other hand, S protein has been demonstrated to augment ingestion of opsonized particles by monocytes (22), such that the adhesive protein may augment phagocytosis of opsonized bacteria by these cells and thus support the immune system of the host organism. Whether certain mechanisms may discriminate between these two pathways initiated by S protein-bacterium interactions awaits further analysis.

ACKNOWLEDGMENTS

This work was supported by grants from Deutsche Forschungsgemeinschaft (Bonn, Federal Republic of Germany).

We are grateful to Gudrun Albohn, Beate Schuster, and Dirk Müller for excellent technical assistance and to Heike Knies for typing the manuscript.

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