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Tissue-Type Plasminogen Activator-Mediated Activation of Plasminogen on the Surface of Group A, C, and G Streptococci

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The interaction of Glu-plasminogen with group A, C, and G streptococci and subsequent formation of surface-associated plasminogen by tissue-type plasminogen activator (t-PA) were studied. Binding of ^{125}I-Glu-plasminogen to streptococci greatly facilitated its activation to ^{125}I-Glu-plasmin by exogenous t-PA, whereas activation in the absence of bacteria took place only slowly. Glu-plasmin formed on the streptococcal surface was further converted to the Lys form. Similar activation and modification took place also in the presence of plasminogen-depleted plasma, containing functional t-PA and plasmin inhibitors, indicating that the surface-associated enzymes were protected against these inhibitors. Lys-plasminogen was 10- to 30-fold more potent than Glu-plasminogen or Glu-plasmin in inhibiting the binding of ^{125}I-Glu-plasminogen to streptococci. This indicated a higher affinity of the Lys form towards plasminogen-binding molecule(s) on the streptococcal surface. The surface-associated plasmin was also enzymically active as judged by digestion of chromogenic substrate S-2251. Surface-associated plasmin activity was observed only when the incubations were carried out in the presence of t-PA and Glu-plasminogen or human plasma as the source of plasminogen. Under these conditions, soluble enzymatic activity was also recovered in the supernatant of group A streptococci. This favors the idea that plasmin can be released from the bacterial surface. The findings provide a mechanism for streptococci to adopt proteolytic activity by binding a host-derived enzyme zymogen on their surface, where the subsequent activation then takes place. The results suggest a role for surface-associated plasmin activity in tissue tropism and tissue invasiveness of streptococci.

Plasminogen, a 91-kDa single-chain glycoprotein, is the key component of the fibrinolytic system. It can be activated to plasmin by activators, such as a tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator, and also by prokaryotic activators, such as staphylokinase and streptokinase. The t-PA-mediated activation takes place poorly in solution but is greatly enhanced by the binding of plasminogen to fibrin or to the eukaryotic cell surface prior to activation. Once bound to solid substratum, plasmin is protected against α_2-antiplasmin and α_2-macroglobulin, which rapidly inactivate the soluble enzyme. Plasmin can further modify itself by cleaving off a small 8-kDa activation peptide from the amino terminus of the molecule; this cleavage converts native Glu-plasminogen to the Lys form (for reviews, see references 5, 17, and 18).

Recently, it has been shown that certain streptococci (20), *Staphylococcus aureus* (8), and certain gram-negative bacteria (16, 19) can bind plasminogen. The binding to bacteria can be inhibited by lysine and its analogs, indicating that the amino-terminal kringle structures of the plasminogen molecule are involved in the interaction (8, 16, 19, 20). By using purified kringle structures and miniplasminogen, the major plasminogen-binding site to streptococci and staphylococci has been located inside kringles 1 to 3 (8, 16, 19).

It has also been reported that plasmin but not plasminogen can bind to certain strains of group A streptococci. Plasmin bound to streptococcal surface was protected from the action of its principal plasma inhibitor, α_2-antiplasmin (4, 10).

In this paper, we have investigated the binding and t-PA-mediated activation of Glu-plasminogen on group A, C, and G streptococci. We report that Glu-plasminogen bound to streptococci can be activated to plasmin by t-PA. Once formed on the surface, plasmin further modifies itself from Glu-plasmin to the Lys form.

MATERIALS AND METHODS

Reagents. Glu- and Lys-plasminogen, Glu-plasmin, and synthetic plasmin substrate S-2251 were purchased from Kabi Vitrum. Plasminogen was also purified from citrated human plasma (Finnish Red Cross Blood Transfusion Center) by affinity chromatography on lysine-Sepharose (6). Plasma absorbed with lysine-Sepharose was used as a plasminogen-depleted plasma. In a double-diffusion immunoprecipitation test, absorbed plasma showed no reactivity against antiplasminogen antiserum (Behringwerke). The concentration of plasminogen in plasma was measured by using the method of Friberger et al. (7). Aprotinin was obtained from Sigma, and single-chain t-PA (containing about 10% of the two-chain form) was obtained from American Diagnostics (New York, N.Y.). For inhibition assays, Glu-plasmin was generated from Glu-plasminogen, as described previously, in the presence of a urokinase-type plasminogen activator and aprotinin (20).

Bacterial strains. Streptococcal strains (A-2947, A-2878, C-13211, and G-975) are described elsewhere (20). Strains were maintained on sealed blood agar plates and transferred every 2 to 4 weeks. Overnight broth cultures of bacterial strains were washed twice and suspended in phosphate-buffered saline (PBS) supplemented with Tween (PBST: 0.12 M NaCl, 14 mM phosphate, 0.05% Tween 20 [pH 7.2]) to a bacterial cell concentration of 5×10^9 cells per ml. For conversion experiments, the bacteria were cultivated in Todd-Hewitt broth without the pH being controlled, as described previously (8), washed twice with PBS (0.01 M

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sodium phosphate, 0.14 M sodium chloride [pH 7.4]) containing 0.02% (wt/vol) sodium azide, and finally suspended in the same buffer. Bacterial concentrations were adjusted spectrophotometrically at an optical density of 640 nm by using a standard curve or an optical density of 36 Klett units for 2×10^8 bacterial cells per ml. Storage of bacterial cells on ice for up to 24 h did not change the results obtained by using fresh bacteria.

Iodination of plasminogen. Iodination of plasminogen was done by using the Iodogen method (13) as described in detail previously (8) or by the chloramine-T method with Iodobeads (Pierce Chemical Company) (20). To eliminate trace amounts of plasmin, purified plasminogen was treated with immobilized aprotinin (Sigma) prior to iodination.

Conversion of plasminogen to plasmin. Conversion of labeled plasminogen on the streptococcal surface was investigated as described previously (8). Briefly, 10 ng of ^{125}I-plasminogen was incubated in 0.5 ml of PBS containing 0.1% (wt/vol) bovine serum albumin (BSA) with 2×10^9 bacterial cells for 2 h at 4°C with aprotinin (100 kallikrein international units [KIU]) in the presence or absence of various amounts of t-PA (50 ng). To monitor the modification of formed plasmin, incubations were also carried out in various combinations in the presence or absence of aprotinin and t-PA. To test the effect of plasma plasmin inhibitors on the conversion, similar incubations were done in the presence of plasminogen-depleted human plasma (100 μl). After incubation, the supernatant was collected, and the cells were washed twice with 2.0 ml of PBS–0.1% BSA and suspended in 100 μl of PBS. Supernatants and cell suspensions were mixed in an equal volume of sample buffer and boiled for 1 min (9). Finally, aliquots (60 μl) were run on a vertical sodium dodecyl sulfate-polyacrylamide (6%) gel electrophoresis (SDS-PAGE) gel under reducing conditions (9) and subsequently analyzed by autoradiography on an X-Omat AR X-ray film (Eastman Kodak Company).

To test the presence of functional plasmin inhibitors in plasminogen-depleted plasma, ^{125}I-Glu-plasminogen (10 ng in 0.5 ml of PBS–0.1% BSA) was treated with plasmin (10 μg) in either the presence or absence of plasminogen-depleted plasma. The digestion end product was then analyzed as described above.

Competition assay. Inhibition assays were performed by incubating 2.5×10^9 bacterial cells in 1.0 ml of PBST buffer supplemented with 20 ng of labeled Glu-plasminogen and 50 KIU of aprotinin per ml together with various amounts of selected competitor (Glu-plasminogen, Glu-plasmin, Lys-plasminogen, or normal human plasma). After 30 min of incubation at room temperature, the bacteria were collected by centrifugation (3,600 × *g* for 15 min), the supernatant was removed, and the radioactivity associated with the bacteria was measured with a gamma counter. The plasminogen uptake was calculated as the percent of the total radioactivity added. The binding percentage without any inhibitor was expressed as 100%.

Plasminogen activation assay. Plasmin activity of surface-bound enzyme was tested as described in detail previously (8). Briefly, 2×10^9 streptococcal cells were incubated for 2 h in 0.5 ml of PBS–0.1% BSA with Glu-plasminogen (25 μg) or human plasma (corresponding to 150 μg of plasminogen) as the source of plasminogen in the presence or absence of t-PA (50 ng). After incubation, the supernatant was collected and the bacteria, after two washes with 2.0 ml of PBS containing 1% BSA (PBS–1% BSA), were suspended in 0.3 ml of PBS–1% BSA. Then, 100-μl aliquots of supernatant and bacterial suspension (corresponding to 7×10^8 cells) were incubated at 37°C for 2 to 6 h in 2.5 ml of PBS containing 80 μM chromogenic substrate S-2251 (H-D-valyl-leucyl-L-lysine-*p*-nitroanilide hydrochloride) and 0.4 M NaCl. The formation of *p*-nitroanilide was recorded spectrophotometrically at a 405-nm wavelength (Utrospec, LKB/Biochrom). Plasmin was used as a positive control. To test the presence of functional plasmin inhibitors in plasma, plasmin was incubated with plasma or plasminogen-depleted plasma prior to incubation with the substrate.

RESULTS

Conversion of plasminogen to plasmin. When ^{125}I-Glu-plasminogen was incubated with group A, C, or G streptococci in the absence of t-PA, the bound plasminogen migrated as a single band in SDS-PAGE corresponding to Glu-plasminogen (Fig. 1, lanes 1 and 3). However, when t-PA was included in the incubation medium, the subsequent SDS-PAGE analysis revealed splitting of the bound plasminogen band into two plasmin bands, heavy (H) and light (L) chains (Fig. 1, lanes 4). Incubation of plasminogen with t-PA without bacteria either in the plain buffer or in the presence of concentrated bacterial culture fluid did not lead to plasmin formation (data not shown).

To study the ability of surface-associated plasmin to further digest and modify itself, conversion experiments were done in the presence or absence of the plasmin inhibitor aprotinin. As seen in Fig. 1 (lanes 4 and 6), the heavy chain of plasmin formed in the absence of aprotinin migrated faster than the one formed when aprotinin was present. There were no differences in the migration of the light chains that were formed.

Analysis of supernatants of bacterial suspensions showed that for all groups of streptococci only the Glu-plasminogen band was seen when the incubations were done in the absence of t-PA (data not shown). In supernatants in which t-PA was present, faint plasmin bands were also detected (data not shown).

To test the effect of plasma plasmin inhibitors on plasminogen activation, activation experiments with plasminogen-depleted human plasma were done. Activation by t-PA was similar for group A, C, and G streptococci in the presence of plasminogen-depleted plasma. The subsequent modification of surface-associated plasmin took place similarly in the plasma milieu and the buffer. The heavy chain of plasmin formed in the absence of aprotinin migrated slightly faster than the heavy chain formed in the presence of aprotinin (Fig. 1, lanes 9 and 11).

To demonstrate the presence of functionally active plasma plasmin inhibitors in plasminogen-depleted plasma, ^{125}I-Glu-plasminogen was incubated with plasmin in either the presence or absence of plasminogen-depleted plasma. Subsequent SDS–6% PAGE and autoradiography analysis revealed that half of the labeled plasminogen treated with plasmin showed a migration pattern similar to that of ^{125}I-Lys-plasminogen (data not shown). Incubation of plasmin with plasminogen-depleted plasma prior to digestion abolished this shift in migration (data not shown).

Inhibition of plasminogen binding. Glu-plasminogen and Glu-plasmin identically inhibited the binding of ^{125}I-Glu-plasminogen to group A, C, and G streptococci (Fig. 2). The concentrations of inhibitor resulting in a 50% displacement of labelled tracer was 100 nM with the group A strain, 50 nM with the group C strain, and 20 nM with the group C strain. Also, normal human plasma containing comparable amounts

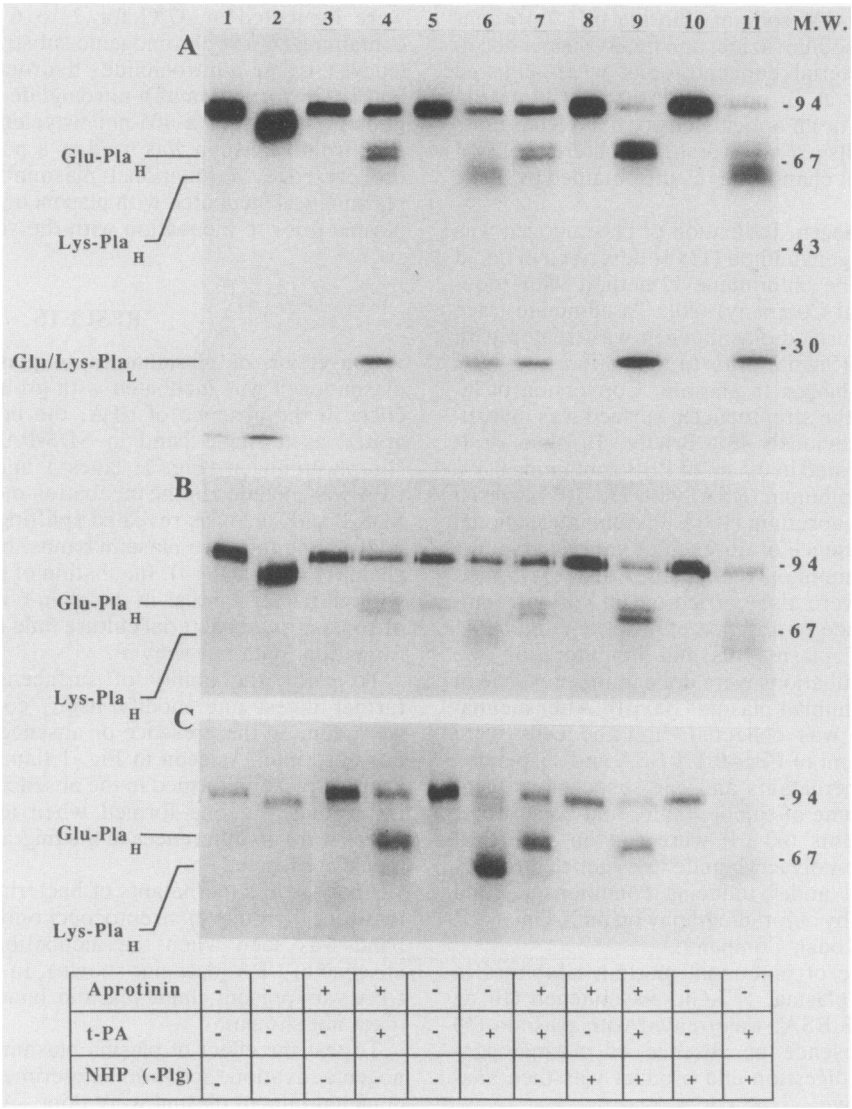

FIG. 1. Binding and activation of Glu-plasminogen on the surface of group A strain A-2974 (A), group C strain C-13211 (B), and group G strain G-975 (C). Streptococci (2×10^9 cells) were incubated with ^{125}I-Glu-plasminogen (10 ng) for 2 h at 4°C in the reaction mixtures containing various combinations of aprotinin (100 KIU) and t-PA (50 ng). Washed bacteria were then analyzed by SDS–6% PAGE and autoradiography. Lanes 1 and 2 show the migration of labeled Glu- and Lys-plasminogen, respectively. For experimental details, see Materials and Methods. The lower portions of the gels for group C and G streptococci are omitted, because they did not differ from that for group A streptococci. At the bottom of the figure, the presence or absence of aprotinin, t-PA, or plasminogen-depleted normal human plasma [NHP(-Plg)] is indicated as plus or minus, respectively. Glu-Pla$_H$, heavy chain of Glu-plasmin; Lys-Pla$_H$, heavy chain of Lys-plasmin; Glu/Lys-Pla$_L$, light chain of Glu- or Lys-plasmin. The migration of molecular weight (MW) markers (phosphorylase *b*, 94,000; BSA, 67,000; ovalbumin, 43,000; and carbonic anhydrase, 30,000) is indicated on the right.

of Glu-plasminogen inhibited the binding, though less efficiently than the purified Glu-plasminogen. This difference was most marked for the group G streptococci.

In all strains, Lys-plasminogen was the most potent inhibitor. Half maximal inhibition was seen at concentrations of Lys-plasminogen 10- to 30-fold lower than those of Glu-plasminogen or Glu-plasmin (Fig. 2).

Plasminogen activation assay. In order to quantitate the surface-associated functional plasmin activity of streptococci, the bacteria were incubated with plasminogen or human plasma in the presence or absence of t-PA. The plasmin activity associated with washed bacterial cells or in the supernatant was then measured by monitoring the breakdown of the synthetic substrate S-2251.

In group A streptococci, the highest plasmin activity was regularly observed with bacteria incubated together with human plasma and t-PA (Fig. 3). With this combination, plasmin activity was also found in the supernatant in spite of the presence of plasma plasmin inhibitors. Bacteria incubated in the plain buffer or in the presence of t-PA alone never showed any plasmin activity. In subsequent experiments, occasional low plasmin activity was seen with bacteria incubated in the absence of t-PA but in the presence of purified Glu-plasminogen or human plasma. Interestingly, incubation in the medium containing both t-PA and Glu-plasminogen rarely led to cell surface-associated plasmin activity. In these cases, supernatants of activation suspension contained respectable enzymatic activity (Fig. 3).

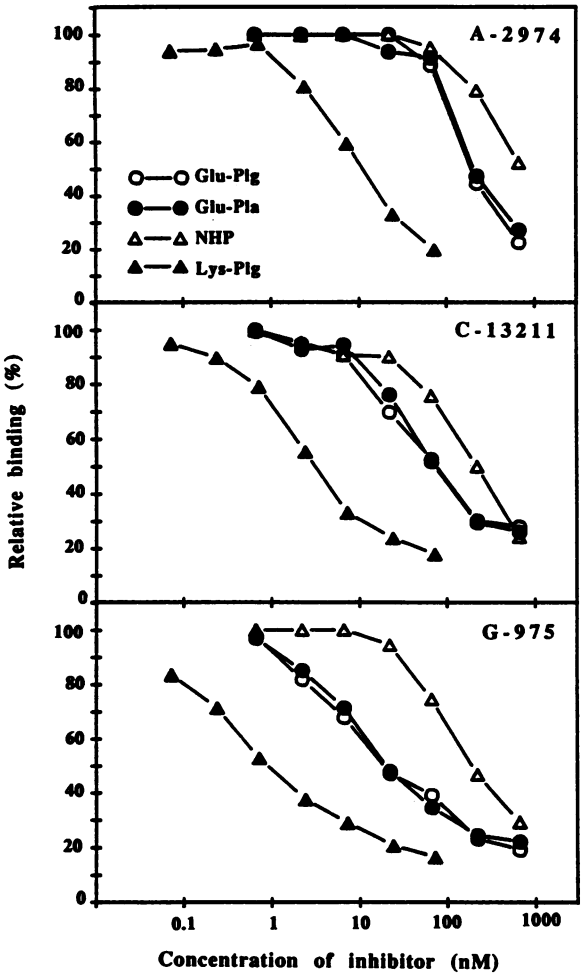

FIG. 2. Inhibition of the binding of Glu-plasminogen to group A, C, and G streptococci by Glu-plasminogen (Glu-Plg), Glu-plasmin (Glu-Pla), normal human plasma (NHP), and Lys-plasminogen (Lys-Plg). Streptococci (2.5×10^9 cells) were incubated for 30 min at room temperature with 20 ng of ^{125}I-Glu-plasminogen in the presence of 50 KIU of aprotinin per ml and the indicated amounts of various inhibitors. The binding percentage without any inhibitor is expressed as 100%. For experimental details, see Materials and Methods.

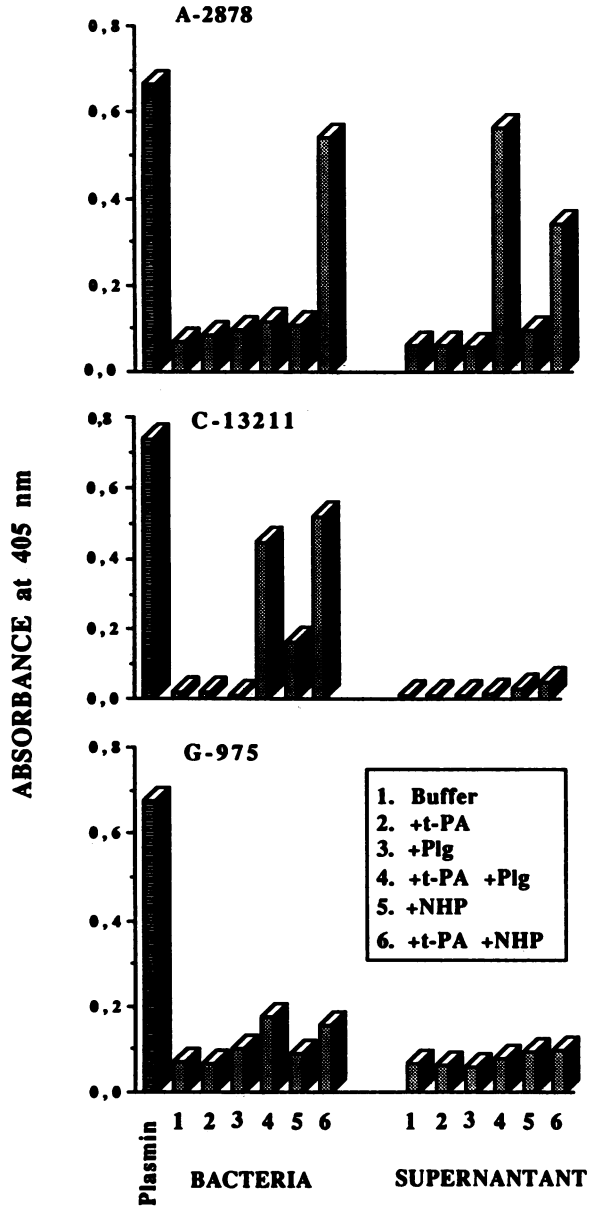

FIG. 3. Plasmin activity associated with group A, C, and G streptococci and found in corresponding supernatants. Streptococci (2×10^9 bacterial cells) were incubated with t-PA (50 ng), Glu-plasminogen (25 μg), and normal human plasma (corresponding to 150 μg of plasminogen) for 2 h at 4°C in the combinations indicated in the inset (Plg, plasminogen; NHP, normal human plasma). After washings, the plasmin activity associated with 7×10^8 bacterial cells or found in bacterial supernatants was measured by monitoring the cleavage of the synthetic chromogenic substrate S-2251. For controls (not shown), the incubation of plasminogen or normal human plasma with t-PA in the absence of bacteria yielded an absorbance of <0.05. Treatment of various amounts of positive control plasmin with normal human plasma prior to the addition of the substrate decreased the absorbance 30 to 80%.

In group C streptococci, the strongest enzymatic activity was found with bacteria which were incubated with t-PA in the presence of Glu-plasminogen or plasma (Fig. 3). No activity was found in the supernatants.

In group G streptococci, low enzymatic activities were found with bacteria incubated with t-PA and either Glu-plasminogen or human plasma. Supernatants contained no enzymatic activity (Fig. 3).

DISCUSSION

Our results confirm the recent findings (20) that plasminogen binds to certain strains of group A, C, and G streptococci. In addition, we demonstrate that surface bound Glu-plasminogen can be activated to Glu-plasmin by t-PA. The observation that no plasmin was formed in the absence of streptococci or concentrated bacterial culture fluid containing released bacterial surface components indicates that the conversion of plasminogen to plasmin really takes place on the cell surface and speaks against the idea that plasmin is first formed in the soluble form and is subsequently bound to the streptococcal surface. In this regard, the mechanism is similar to the activation demonstrated for *S. aureus* (8).

It is important to note that plasmin once formed on the streptococcal surface is also active in the presence of functional plasma plasmin inhibitors. This is in agreement with the finding of Lottenberg et al. (10) who demonstrated that exogenous plasmin bound to group A streptococci was protected against the inactivation by α_2-antiplasmin inhibitor. They showed, however, that binding of the inhibitor to soluble plasmin prevented its binding to streptococci. In light of these results, it seems unlikely that streptococci would adopt surface-associated plasmin activity via binding of soluble enzyme which, in physiological fluids, most probably is bound to α_2-antiplasmin. A more likely mechanism is the binding and subsequent activation of plasminogen on the streptococcal surface.

The surface-associated plasmin has the capability to further modify itself from Glu to Lys form by releasing the activation peptide from the amino-terminal end of the molecule. This modification is known to cause conformational changes in the kringle region of plasminogen (11, 12, 18). According to our results, Lys-plasminogen was 10- to 30-fold more potent than Glu-plasminogen and Glu-plasmin in inhibiting the binding between streptococci and Glu-plasminogen. This suggests that Lys-plasminogen has a higher affinity towards the plasminogen-binding component(s) on the streptococcal surface. In our assay, normal human plasma containing intact plasminogen inhibited plasminogen binding to streptococci less effectively than did purified Glu-plasminogen. The molecular background for this event is presently poorly understood, but it would result from binding of other plasma proteins to streptococcal surface and interaction in the binding assay. With *S. aureus*, it was shown that the modification from Glu- to Lys-plasminogen increased the affinity roughly 100-fold (8). This observation also agrees with the earlier finding that Glu-plasminogen was a poor inhibitor of the binding between group A streptococci and Lys-plasminogen (2). Since Lys-plasminogen is only a transient form of plasminogen, the finding favors the idea that Lys-plasmin could also bind more tightly to the streptococcal surface. This would have an important physiological consequence by preventing Glu-plasminogen, which is present in circulation and many physiological fluids, from releasing the surface-associated active enzyme. In this manner, streptococci would retain the surface-associated plasmin activity without the need to constantly activate new zymogen.

Plasmin formed on the streptococcal surface also has functional enzymatic activity as shown by digestion of chromogenic substrate S-2251. The highest activity was always associated with bacteria incubated in the presence of t-PA and normal human plasma as the source of plasminogen. This again demonstrates that the binding and activation of Glu-plasminogen can take place in the presence of functional plasma protease inhibitors. Substitution of plasma with purified Glu-plasminogen also leads to surface-bound plasmin activity with groups C and G, but not with group A, streptococci. Similar to the results from conversion experiments with these combinations, plasmin activity was found in the supernatants of group A streptococcus activation suspension. Because plasminogen activation by t-PA occurs very poorly in plain buffer or in spent media of bacterial cultures, this finding indicates that the active enzyme is released from the bacterial surface by a mechanism which is not understood. It is difficult to estimate the in vivo importance of the release. The bacteria were grown to stationary phase, and perhaps the release of the enzyme is due only to the destruction of dead bacterial cells. However, the finding raises a tempting possibility that bacterial cells can shed receptor-bound plasmin, thus providing soluble active enzyme which is protected against plasmin inhibitors in plasma.

In some experiments, low plasmin activity was also observed with streptococci, especially groups A and C, incubated in the presence of normal human plasma alone. This can be explained by the presence of endogenous t-PA in plasma. Storage of plasma will increase the binding of t-PA with its principal inhibitor, PAI I, leading to lower functional t-PA levels in some plasma samples.

The role of streptokinase as an activator of surface-bound plasminogen is interesting. Streptokinase activates plasminogen by forming a complex with plasmin or plasminogen which subsequently can activate plasminogen (1, 14). It has been suggested that this ability might have a role for the invasive properties of many streptococcal infections. In several experiments, concentrated medium of streptococcal cultures, supposedly containing soluble streptokinase, was unable to activate plasminogen as judged by conversion of ^{125}I-plasminogen to ^{125}I-plasmin. However, incubation of living bacteria, either at 4°C or 20°C, with plasminogen was occasionally associated with weak surface-associated plasmin activity. In our streptococcal culture, the pH of the culture medium was not controlled, and this may have the effect of streptokinase degradation. Therefore, a weak surface-associated plasmin activity in some streptococcal preparations may result from the presence of low amounts of streptokinase. This matter, however, has to be studied further in a more detailed set of experiments. Our present results focus on t-PA-mediated plasminogen activation on the streptococcal surface without excluding the possibility that streptokinase would have an in vivo activation role. Very recently, Broder et al. have described a 41-kDa plasmin-binding protein originating from a mutanolysin digest of a group A streptococcal strain. The molecule is antigenically and functionally different from streptokinase produced by other group A and C streptococci (3). Its presence on the surface of an intact group A streptococcal cell remains, however, to be elucidated.

The results presented in this paper widen the novel concept of how bacteria can adopt surface-associated proteolytic activity (8). Together with the earlier findings (20), the data imply that streptococci will retain Glu-plasminogen on their surfaces where the proenzyme then can be activated via plasminogen activators. In this respect, the results are very similar also to those reported very recently that S-fimbriated *Escherichia coli*, known to cause neonatal septicemia and meningitis, can bind plasminogen and express t-PA-mediated plasminogen activation via the fimbria (15).

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