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Receptors for Human Plasminogen on Gram-Negative Bacteria

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A total of 188 strains representing 11 species of gram-negative bacteria were examined for the ability to interact with human plasminogen. Highly purified human plasminogen was labeled with ^{125}I, and its uptake by different bacterial strains was measured. All 14 strains of *Haemophilus influenzae* and all 13 strains of *Branhamella catarrhalis* tested were positive with respect to plasminogen uptake. Also, eight species belonging to the family *Enterobacteriaceae* were tested, and of those, *Proteus mirabilis* demonstrated the most substantial uptake, with 28 of 39 strains taking up more than 10% of the plasminogen. Ten strains of *Pseudomonas aeruginosa* were also tested, of which seven showed uptake values higher than 10%. With *H. influenzae* and *B. catarrhalis* strains, Scatchard analysis indicated a two-phase receptor interaction, one more-avid receptor with a K_d of 6 to 8 nM and 2,000 to 2,500 sites per bacterium and a second receptor with a K_d of 50 to 80 nM and 9,000 sites per bacterium. With *Pseudomonas aeruginosa* strains, a single receptor interaction was detected with a K_d of 60 nM and the number of sites was estimated as 8,000 per bacterium. Scatchard analysis with strains of *P. mirabilis* indicated binding of a less-specific nature. However, plasminogen uptake by this species could be reduced by 50% by the addition of 2 mM unlabeled plasminogen. This estimate of K_d, as well as uptake studies with plasminogen fragments, suggests different properties of this receptor. With all receptor types, the addition of plasmin-aprotinin complex inhibited plasminogen uptake, which demonstrates that both forms of the molecule react with the same receptors. Plasminogen uptake could be eliminated by the addition of lysine or ε-aminocaproic acid, which suggests that the lysine-binding sites of the plasminogen molecule are involved in the receptor-ligand interaction.

Plasminogen, a glycoprotein with a molecular weight of 92,000, plays a central role in hemostasis as the main mediator of fibrinolysis. The molecule is inactive in its native form but is converted to the active form, plasmin, by the split of a single peptide bond. The resulting A and B chains remain connected by two disulfide bridges. The A chain consists of five triple-loop structures (kringles) with pronounced internal homology (13). These kringles carry lysine-binding sites responsible for the binding to fibrin and alpha$_2$-antiplasmin. The active site, on the other hand, is located on the B chain. Receptors for plasminogen have been detected on all types of leukocytes and platelets, as well as on other types of somatic cells (8). In recent investigations, we have been able to demonstrate specific receptors for plasminogen on several species of gram-positive cocci, including hemolytic group A, C, and G streptococci and pneumococci (12). In this paper, we present evidence for the existence of receptors for plasminogen on several unrelated species of gram-negative bacteria as well.

MATERIALS AND METHODS

Bacterial strains and media. A total of 188 bacterial strains of 11 different gram-negative species were examined. Strains included *Escherichia coli* (8 strains), *Klebsiella pneumoniae* (36 strains), *Klebsiella oxytoca* (10 strains), *Proteus mirabilis* (39 strains), *Proteus vulgaris* (32 strains), *Citrobacter freundii* (11 strains), *Enterobacter cloacae* (9 strains), *Enterobacter agglomerans* (6 strains), *Pseudomonas aeruginosa* (10 strains), *Haemophilus influenzae* (14 strains), and *Branhamella catarrhalis* (13 strains). The strains were collected from fresh clinical laboratory specimens. Strains of *H. influenzae* and *B. catarrhalis* were cultivated in Todd-Hewitt broth supplemented with 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) and 40 mg of hemin (Difco Laboratories, Detroit, Mich.) per liter. All other bacteria were cultivated in Luria-Bertani broth. Strains were kept frozen in broth supplemented with 20% glycerol at −70°C and recultivated 2 to 3 days prior to testing. Repeated freezing and thawing of bacterial strains did not affect plasminogen uptake levels.

Plasminogen preparations. Highly purified plasminogen with a relative plasmin concentration of <10 ppm was prepared according to procedures described previously (16). No impurity could be found by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Plasminogen fragments. Plasminogen fragments were obtained by elastase digestion essentially as described by Sottrup-Jensen et al. (11). However, we used elastase bound to Sepharose, which could be conveniently removed by gel filtration after digestion. The digestion procedure results in three major fragments: kringle 1–3, kringle 4, and miniplasminogen (kringle 5 and the B-chain portion). These were separated by lysine-Sepharose chromatography and gel filtration on Sephadex G-75 as previously described (11).

Radiolabeling of plasminogen and plasminogen fragment preparations. Plasminogen preparations were labeled with ^{125}I (Radiochemical Centre, Amersham, England) by the chloramine-T method with Iodo-beads (Pierce Chemical Co., Rockford, Ill.). Plasminogen or plasminogen fragment (100 μg) in 0.1 ml of phosphate-buffered saline (0.12 M NaCl, 14 mM phosphate; pH 7.2) was mixed with 0.2 mCi of ^{125}I at room temperature, and an Iodo-bead was added. After 5 min of incubation, the bead was removed. The labeled protein was separated from free ^{125}I by gel filtration on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). Labeled plasminogen was diluted to an appropriate concentration, stabilized by the addition of aprotinin (Bayer AG, Leverkusen,

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Federal Republic of Germany) at a concentration of 100 kIU/ml, and kept frozen at −70°C. The specific activities of labeled plasminogen and plasminogen fragments were approximately 400 cpm/ng. Dodecyl sulfate-polyacrylamide gel electrophoresis prior to and after reduction confirmed that no degradation of plasminogen had occurred. The content of Lys-plasminogen was less than 2%.

Plasmin-aprotinin complex. Plasminogen (10 mg) was dissolved in 2 ml of phosphate-buffered saline supplemented with 1 mM 6-aminohexanoic acid (E. Merck AG, Darmstadt, Federal Republic of Germany). Aprotinin was added (5,000 kIU/ml) to block active sites on plasmin molecules, 1,000 Plough units of urokinase (Leo Pharmaceutical Products, Ballerup, Denmark) was added, and activation was allowed to proceed overnight at 0°C. Dodecyl sulfate-polyacrylamide gel electrophoresis confirmed that the activation was complete.

Plasminogen-binding assay. Overnight broth cultures of bacterial strains were washed twice and suspended in phosphate-buffered saline supplemented with Tween 20 (0.12 M NaCl, 14 mM phosphate, 0.05% Tween 20; pH 7.2) to a bacterial concentration of 5×10^9/ml. Bacterial counts were calculated from optical density values at 620 nm on diluted samples in an LKB 540 colorimeter. Binding studies were performed at room temperature with disposable polystyrene test tubes and 2.5×10^9 bacteria in each test. Bacteria were suspended in buffer containing 50 kIU of aprotinin per ml to prevent activation. Labeled plasminogen (20 ng) was added to each tube. After 30 min of incubation, the bacteria were collected by centrifugation at 3,600 × *g* for 15 min. The supernatant was removed, and the radioactivity in the pellet was measured with a gamma counter. The plasminogen uptake was expressed as the percentage of the total radioactivity added. Uptake values above 10% were considered positive. Dissociation constants were estimated from competitive blocking experiments utilizing unlabeled plasminogen or plasmin-aprotinin complex. The concentration of unlabeled ligand causing a 50% reduction of bound tracer was taken as an estimate of K_d (15). This approximation is valid provided that the concentration of the labeled ligand is much lower than the concentration of the receptor. In all competitive binding experiments, the receptor concentration exceeded the concentration of labeled plasminogen by more than 2 orders of magnitude.

Scatchard analysis. A dilution series of labeled plasminogen (20 ng/ml to 10 μg/ml) was added to 2.5×10^9 bacteria by the procedure described above. Experiments were performed in triplicate. Nonspecific binding was also measured after the addition of 10 μM unlabeled plasminogen as blocking agent. Specific binding was calculated by subtracting nonspecific binding from total binding. Nonspecific binding was at the same level as that seen with nonreactive *E. coli* ATCC 25922, i.e., around 3%. The ratio of bound to free plasminogen was plotted as a function of bound plasminogen by Scatchard analysis (10). Linear regression analysis was used for curve fitting, with the slope represented as $-1/K_d$ and the *x* intercept giving the total number of receptors.

RESULTS

Plasminogen binding to gram-negative bacteria. A total of 188 bacterial strains were tested for uptake of ^{125}I-labeled plasminogen. Individual results for 6 of 11 species are plotted in Fig. 1. All 14 strains of *H. influenzae* and all 13 strains of *B. catarrhalis* were positive with respect to plasminogen uptake, with values ranging from 42 to 68 and 30 to 56%, respectively. Eight species belonging to the family *Enterobacteriaceae* were tested, of which *P. mirabilis* demonstrated the most substantial plasminogen uptake, with values ranging from 6 to 78% and with values for 28 of 39 strains higher than 10%. The related species *P. vulgaris* had a maximum uptake of 21%, with uptake for 7 of 32 strains higher than 10%. All strains of *E. coli*, *K. pneumoniae*, and *K. oxytoca* were below or around the 10% level. Strains of *C. freundii*, *E. cloacae*, and *E. agglomerans* formed homogeneous clusters with median values of 11% and with no strain with values exceeding 17%. Plasminogen binding to *Pseudomonas aeruginosa* was also tested. Of 10 strains tested, 7 demonstrated uptake values above the 10% level, with a maximum uptake of 39%.

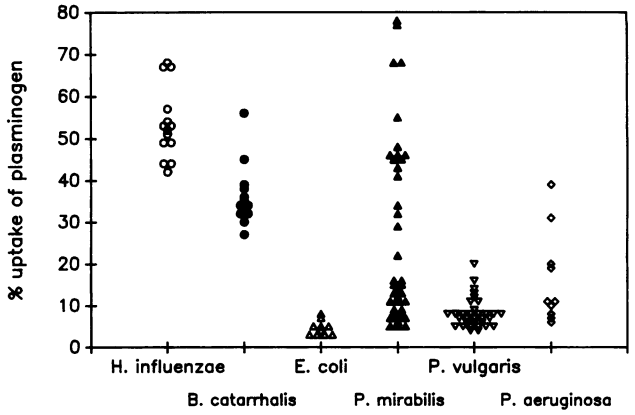

FIG. 1. Binding of ^{125}I-labeled plasminogen to six species of gram-negative bacteria. Bacterial cultures were incubated with plasminogen for 30 min. Binding is expressed as percent uptake of total plasminogen added.

Scatchard analysis of receptor interaction. Scatchard analysis was used to estimate K_d values and receptor numbers on selected bacterial strains. The results are summarized in Fig. 2. Both *H. influenzae* HI-23459 and *B. catarrhalis* BC-992 displayed a two-phase interaction, which indicates the possibility of two different receptor structures. With HI-23459, K_d values were 8 nM (2,000 receptors per bacterium) for phase 1 and 80 nM (9,000 receptors per bacterium) for phase 2. The corresponding values for BC-992 were 6 nM (2,500

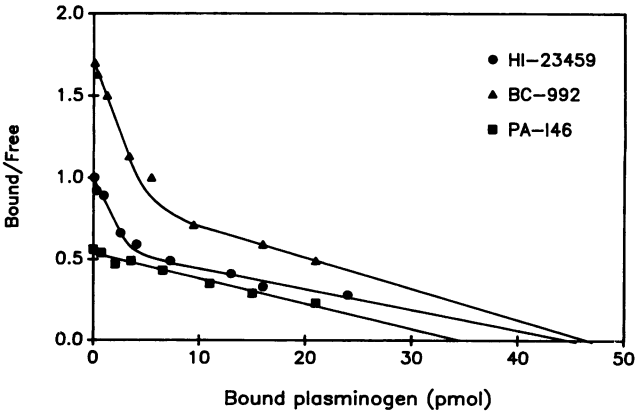

FIG. 2. Scatchard analysis of plasminogen interaction with three bacterial species. Labeled plasminogen was added to bacterial cultures and allowed to incubate for 30 min. Experiments were performed in triplicate, and mean values are plotted.

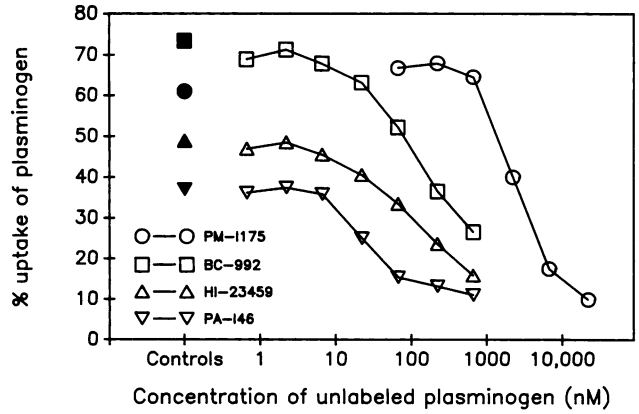

FIG. 3. Inhibition of ^{125}I-labeled plasminogen uptake with unlabeled plasminogen. Labeled and unlabeled plasminogen were added simultaneously to bacterial cultures and allowed to incubate for 30 min. Percent uptake of labeled plasminogen is expressed as a function of inhibitor concentration. Controls: ■, BC-992; ●, PM-I175; ▲, HI-23459; and ▼, PA-I46.

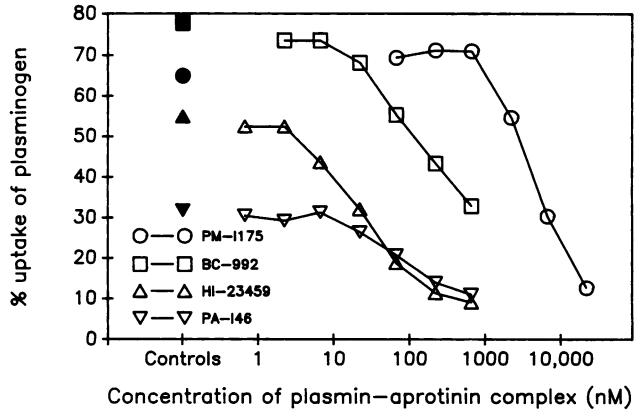

FIG. 4. Inhibition of ^{125}I-labeled plasminogen uptake with plasmin-aprotinin complex. Labeled plasminogen and inhibitor were added simultaneously to bacterial cultures and allowed to incubate for 30 min. Percent uptake of labeled plasminogen is expressed as a function of inhibitor concentration. Symbols are as described in the legend to Fig. 3.

receptors per bacterium) and 50 nM (9,000 receptors per bacterium). Analysis of *Pseudomonas aeruginosa* PA-I46 demonstrated a single-phase interaction with a K_d of 60 nM and 8,000 receptors per bacterium. Scatchard analysis was also performed with strains of *P. mirabilis* and resulted in almost horizontal plots, indicating a nonspecific interaction (data not shown).

Competition with unlabeled plasminogen and plasmin-aprotinin complex. As an alternative way of estimating the K_d, competitive blocking experiments with unlabeled plasminogen were performed. Figure 3 summarizes results from experiments with selected strains of four species. With all four species, the addition of unlabeled plasminogen efficiently blocked the uptake of tracer. With *H. influenzae* HI-23459, *B. catarrhalis* BC-922, and *Pseudomonas aeruginosa* PA-I46, the concentrations causing a 50% reduction of bound tracer were in the range of 40 to 200 nM. With *P. mirabilis* PM-I175, the required concentration was 2 mM.

To examine whether plasminogen-binding receptors are also capable of binding plasmin, blocking experiments with plasmin were performed. As plasmin itself is a very unstable molecule, plasmin-aprotinin complex was chosen as the inhibiting agent. Figure 4 summarizes the results with the same four strains. With *H. influenzae* HI-23459, *Pseudomonas aeruginosa* PA-I46, and *B. catarrhalis* BC-922, the concentrations causing a 50% reduction of bound tracer were in the range of 40 to 150 nM, whereas 4 mM was needed with *P. mirabilis* PM-I175.

Blocking with lysine or EACA. To elucidate whether the lysine-binding sites on the plasminogen molecule are involved in the receptor interaction, blocking experiments with lysine and ε-aminocaproic acid (EACA) were performed. The results are summarized in Fig. 5. Both agents efficiently blocked plasminogen uptake. The concentrations needed for a 50% reduction of bound tracer were approximately 1 mM for lysine and 0.03 to 0.1 mM for EACA.

Binding of plasminogen fragments. In an attempt to examine which domains of the plasminogen molecule interact with the different bacterial receptors, ^{125}I-labeled fragments of the molecule were used. Three fragments, including kringle 1–3, kringle 4, and miniplasminogen (kringle 5 and the B chain), were tested, and the results were compared with those for native plasminogen (Table 1). Values are presented as uncorrected uptake (A) and corrected uptake, i.e., with binding in the presence of 100 mM EACA subtracted (B). *E. coli* ATCC 25922 was used as a negative control. This strain did not interact substantially with native plasminogen or any fragment. All three strains of *H. influenzae* demonstrated a limited but significant uptake of kringle 1–3. A slight uptake of miniplasminogen was also detected. All strains of *B. catarrhalis* reacted with both kringle 1–3 and miniplasminogen but at levels lower than those with native plasminogen. *Pseudomonas aeruginosa* PA-I46 demonstrated uptake of kringle 1–3 only. *P. mirabilis* strains clearly differed from strains of other species with respect to fragment pattern. All strains demonstrated uptake values of kringle 1–3 comparable to those of native plasminogen. Two strains also bound substantial amounts of kringle 4.

DISCUSSION

Receptors for plasma proteins have been described for several species of bacteria. On gram-positive cocci, binding structures have been defined for about 10 different proteins. Also, gram-negative bacteria have been demonstrated to carry receptors for plasma proteins. Thus, receptors for fibronectin have been detected on enterotoxigenic strains of *E. coli* (3) as well as on strains of *Salmonella enteritidis* (1). Adhesins of some uropathogenic *E. coli* strains have also been demonstrated to react with laminin (14).

In this study, we demonstrate the occurrence of receptors for plasminogen on gram-negative bacteria representing a wide range of species. All strains of *H. influenzae* and all strains of *B. catarrhalis* tested demonstrated a distinct uptake of plasminogen. With *Pseudomonas aeruginosa*, about half of the strains were positive. Of the eight species tested belonging to the family *Enterobacteriaceae*, the highest uptake was demonstrated with *P. mirabilis*. A few strains of the related species *P. vulgaris* also reacted with plasminogen at levels that could be interpreted as significant, whereas *E. coli*, *K. pneumoniae*, and *K. oxytoca* were negative. The three other species demonstrated a slight uptake, the specificity of which has not yet been determined. Scatchard analysis demonstrated receptor-ligand interactions of high specificity for *H. influenzae*, *B. catarrhalis*, and *Pseudomonas aeruginosa*, and the number of receptors was estimated as about 10^4 per bacterium. With *P. mirabilis*, the

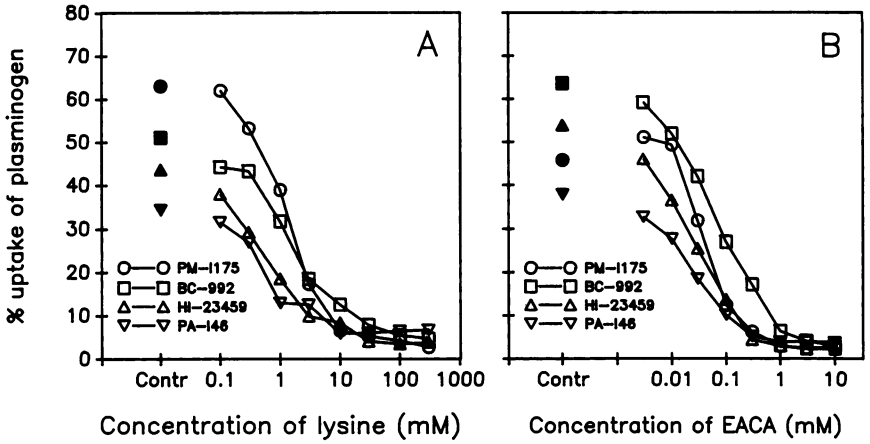

FIG. 5. Inhibition of ^{125}I-labeled plasminogen uptake with lysine (A) and EACA (B). Labeled plasminogen and inhibitor were added simultaneously to bacterial cultures and allowed to incubate for 30 min. Percent uptake of labeled plasminogen is expressed as a function of inhibitor concentration. Symbols are as described in the legend to Fig. 3.

reaction seemed more unspecific. Competitive blocking experiments with plasmin-aprotinin demonstrated that all receptor types reacted equally well with plasmin. Lysine or EACA also completely blocked plasminogen uptake, which suggests that the lysine-binding sites on the plasminogen molecule are involved in the receptor-ligand interaction.

Recently, we were able to demonstrate a similar type of receptor on several species of gram-positive cocci (12). These included beta-hemolytic group A, C, and G streptococci, pneumococci, and some strains of alpha-hemolytic streptococci. The affinities of those receptors were of the same magnitude as the affinities of receptors on gram-negative bacteria described here. The number of receptors per bacterium was also estimated as about 10^4 (unpublished data). Plasmin-aprotinin complex, could competitively block the uptake of plasminogen, which suggests that it binds to the same receptor. This receptor is probably not related to streptokinase, as our results indicated an interaction mainly with kringle 1–3, whereas streptokinase is known to interact with the B-chain portion. A receptor similar to the plasminogen-plasmin-binding protein described here but with very high affinity for plasmin only has also been found on group A streptococci (2, 5).

Receptors for plasminogen have been detected on several types of eucaryotic cells, including platelets (6), monocytes, granulocytes, and T and B lymphocytes (7). Generally, these receptors seem to bind plasminogen and plasmin with equal affinity (9), but some investigators report a higher affinity for plasmin than for plasminogen (4). Our Scatchard analysis and competitive blocking experiments suggest that the affinity for the bacterial receptor on *H. influenzae*, *B. catarrhalis*, and *Pseudomonas aeruginosa* is 1 to 2 orders of magnitude higher than for the corresponding receptor on eucaryotic cells, which makes it unlikely that the interaction is merely a nonspecific phenomenon. The dissociation constants are similar to those for gram-positive cocci, but

TABLE 1. Uptake of ^{125}I-labeled plasminogen fragments

Bacterial species and strains	% Uptake[a]							
	Plasminogen		Kringle 1–3		Kringle 4		Kringle 5 with B chain (miniplasminogen)	
	A	B	A	B	A	B	A	B
Haemophilus influenzae								
HI-23426	51.8	49.4	10.3	7.7	4.7	1.7	10.9	6.6
HI-23459	38.5	34.0	7.1	5.0	5.3	0.0	6.9	3.4
HI-23423	32.8	30.0	6.6	4.4	4.9	0.0	5.7	2.2
Branhamella catarrhalis								
BC-992	57.8	54.3	11.5	8.4	4.9	1.3	15.6	6.2
BC-2043	30.1	27.5	9.6	4.6	4.9	0.5	14.9	5.2
BC-12	38.7	34.9	10.6	7.8	5.5	2.1	12.7	7.4
Proteus mirabilis								
PM-I175	56.8	53.9	51.2	49.8	4.7	0.0	2.5	0.0
PM-U-35	66.5	56.3	60.0	58.0	14.4	7.6	4.9	2.3
PM-I22	25.3	23.3	15.7	14.0	9.1	5.8	3.7	0.0
Pseudomonas aeruginosa PA-I46	27.3	21.9	11.3	8.1	4.1	0.6	6.2	2.3
Escherichia coli ATCC 25922	4.1	0.3	2.8	0.0	2.9	0.2	1.8	0.0

[a] Values are presented as uncorrected uptake (A) and corrected uptake, with binding in the presence of 100 mM EACA subtracted from total uptake (B).

whether these receptors are structurally related remains to be elucidated. Compared with other receptors, the plasminogen receptor on *P. mirabilis*, on the other hand, demonstrated quite different properties in fragment-binding experiments. Scatchard analysis suggested a less-specific interaction, but in competitive blocking experiments, the addition of 2 mM unlabeled plasminogen resulted in a 50% reduction of bound tracer. This estimate of K_d, however, indicates an affinity slightly lower than that of the eucaryotic receptor, which implies that this receptor could still have a biological function. This receptor is obviously different from those detected on other bacteria.

Different functions for the plasminogen receptors on eucaryotic cells have been postulated (8). One function could be a local enrichment of the molecule within the inflammatory process. Experimental data also suggest that these receptors facilitate activation of plasminogen and protect it from inactivation by alpha$_2$-antiplasmin (8). Such functions could be of benefit for the migration of inflammatory cells through fibrin barriers and would also constitute a plausible reason for the existence of such receptors on an invading microorganism.

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