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Degradation of Soluble Laminin and Depletion of Tissue-Associated Basement Membrane Laminin by *Pseudomonas aeruginosa* Elastase and Alkaline Protease

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Purified *Pseudomonas aeruginosa* elastase and alkaline protease rapidly cleaved soluble laminin, with each enzyme yielding different cleavage products. These cleavage fragments were separated from the intact laminin A and B polypeptide chains by sodium dodecyl sulfate-polyacrylamide gradient gel electrophoresis and detected by their characteristic Coomassie blue staining patterns. Pseudomonas elastase produced rapid and extensive degradation of both A and B chains, including the disulfide-rich regions. Apparently complete degradation to limit digests was obtained after 30 min with a substrate/enzyme ratio of 30:0.5. Under similar conditions, alkaline protease rapidly degraded the A chain while slowly degrading the B chain. In addition, immunoreactive laminin was released from authentic basement membranes after incubation with either enzyme as detected by an enzyme-linked immunoabsorption assay and by immunofluorescence. The results from these studies suggest a direct role for elastase and alkaline protease in both tissue invasion and hemorrhagic tissue necrosis in *P. aeruginosa* infections.

A characteristic feature of *Pseudomonas aeruginosa* infections is tissue invasion associated with extensive necrosis, microabscess formation, and destructive vascular lesions with hemorrhage (33). This organism is a common cause of hospital-acquired, gram-negative, necrotizing pneumonias in the United States (32) and is a major pathogen in patients with cystic fibrosis, burns, and neutropenia (5). Despite intensive study, the pathogenesis of *P. aeruginosa* tissue invasion is imperfectly understood. However, most strains of *P. aeruginosa* produce extracellular proteases that may be important for tissue invasion and necrosis (13, 18, 22). For example, pseudomonas elastase (PE) proteolytically inactivates certain components thought to be important in initiating and modulating inflammatory responses such as complement components and complement-derived peptides (34), alpha-1-proteinase inhibitor (25, 28), immunoglobulin G (IgG) (7, 8, 12), IgA (7), and airway lysozyme (14). Recently, we demonstrated that PE degrades human types III and IV collagens with the formation of specific cleavage fragments (10). Degradation of these collagen types could explain a role for this enzyme in both infectivity and hemorrhagic necrosis. Another extracellular matrix glycoprotein, laminin, is the major noncollagenous component of all basement membranes (16, 36). Besides binding specifically to other basement membrane components such as heparan sulfate proteoglycans (16, 38) and type IV collagen (35), laminin promotes adhesion of various cell types to the basal lamina (16). The principal findings of the present study are that PE and pseudomonas alkaline protease (PAP) cleave purified soluble laminin and deplete tissue-associated basement membrane laminin.

MATERIALS AND METHODS

Preparation of laminin and anti-laminin IgG. Laminin was purified from the Englebreth-Holm-Swarm murine sarcoma by sequential salt extraction and ion-exchange and gel filtration chromatography by a modification of the method of Timpl et al. (36) and characterized as previously described (2). Laminin was quantitated by the Lowry et al. procedure (20) with bovine serum albumin as the protein standard and stored at 4°C in 0.5 M NaCl–0.05 M Tris hydrochloride (pH 7.6) containing 0.01% sodium azide.

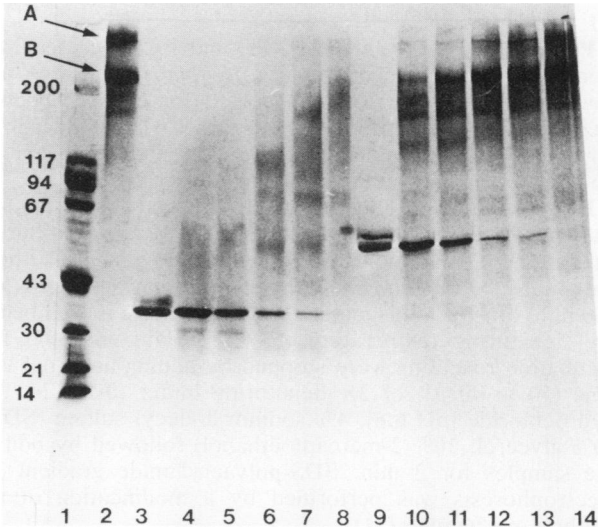

FIG. 1. Coomassie blue staining of SDS-polyacrylamide gradient gel electrophoresis run under reducing conditions after incubating various amounts of PE or PAP with constant amounts of laminin at a fixed time and temperature. In lane 1 are the globular protein molecular weight standards, and their approximate $M_r \times 1{,}000$ are shown on the left. In lane 2 is 30 μg of laminin, and its A and B chains are shown. In lanes 3 and 9 are 10 μg of PE and PAP, respectively. In lanes 4 through 8 are 30 μg of laminin incubated with 10, 5, 1, 0.5, and 0.1 μg of PE, respectively. In lanes 10 through 14 are 30 μg of laminin incubated with 10, 5, 1, 0.5, and 0.1 μg of PAP, respectively. All samples in lanes 2 through 14 were incubated for 30 min at 37°C.

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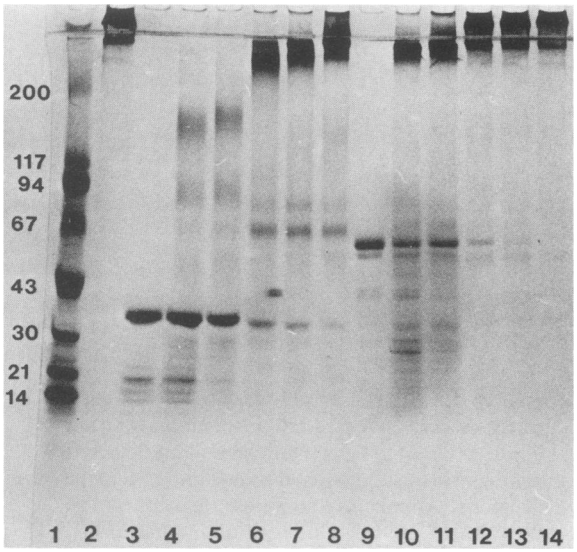

FIG. 2. Coomassie blue staining of SDS-polyacrylamide gradient gel electrophoresis run under nonreducing conditions after incubating various amount of PE or PAP with constant amounts of laminin at a fixed time and temperature. In lane 1 are the globular protein molecular weight standards. In lanes 2, 3, and 9 are 30 μg of laminin and 10 μg of PE and PAP, respectively. In lanes 4 through 8 are 30 μg of laminin incubated with 10, 5, 1, 0.5, and 0.1 μg of PE, respectively. In lanes 10 through 14 are 30 μg of laminin incubated with 10, 5, 1, 0.5, and 0.1 μg of PAP, respectively. All samples in lanes 2 through 14 were incubated for 30 min at 37°C.

Affinity-purified sheep and rabbit anti-laminin IgGs were eluted from laminin-Sepharose columns (2, 3) and characterized by an enzyme-linked immunoabsorbant assay as before (1).

Preparation of PE and PAP. PE and PAP were isolated from the nutrient media of *P. aeruginosa* cultures and characterized as previously reported for each respective enzyme (21, 27). The lyophilized enzymes were standardized for protein content, assayed for elastinolytic and caseinolytic activity, and stored as previously described (10).

Degradation of soluble laminin by PE and PAP. Laminin was incubated with or without PE or PAP at different substrate/enzyme ratios (300:1 to 3:1) for 30 min at 37°C or at the same substrate/enzyme ratio (30:1) for various times (5 to 480 min) at 37°C. This incubation temperature is well below the denaturing temperature (58°C) of laminin (30). The incubation reactions were stopped by adding an equal volume (50 to 70 μl) of 2× denaturing buffer (0.125 M Tris hydrochloride [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% 2-mercaptoethanol) followed by boiling the samples for 2 min. SDS-polyacrylamide gradient gel electrophoresis was performed by a modification of the method of Laemmli (11).

Tissue degradation assays. Kidneys from adult Sprague-Dawley rats were used for both assays because they are a rich source of basement membranes. First, an enzyme-linked immunosorbent assay was developed. Three 10-μm-thick cryostat sections of unfixed rat kidney cortex were placed into each of a series of microfuge tubes (1.5 ml). Solutions (1 ml) of either PE or PAP (0.05 to 5 μg/ml) in 0.05 M Tris hydrochloride (pH 7.6) containing 0.15 M NaCl and 0.001 M $CaCl_2$ were then added, and the tubes were incubated for 1 h at 37°C. The digestion was stopped with the addition of EDTA to a final concentration of 0.001 M, and the tubes were centrifuged at 7,000 × *g* for 5 min. As controls, sections were incubated for 1 h with buffer alone followed by the addition of EDTA. Supernatants from the enzyme- and buffer-treated sections were placed in microtiter wells overnight at 4°C. Samples (50 μl) of 0.8 μg of affinity-purified rabbit anti-laminin IgG were then added to the plates and incubated. After washing three times, the plates were treated with goat anti-rabbit IgG-horseradish peroxidase (Cooper Biomedical, Malvern, Pa.) and developed for peroxidase activity (6). Second, 6-μm-thick cryostat sections were placed on glass slides and air dried. Sections were flooded for 2 min at 25°C with 0.25 μg of either PE or PAP per ml or with buffer. After washing, sections were treated sequentially with affinity-purified sheep anti-laminin IgG and fluorescein-rabbit anti-sheep IgG (Cooper Biomedical). Slides were then examined by epifluorescence microscopy.

RESULTS

Enzymatic cleavage of soluble laminin. On SDS-polyacrylamide gradient gels, laminin migrated as polypeptides A (M_r ~440 × 10^3) and B (M_r ~200 × 10^3 to 220 × 10^3) after reduction (Fig. 1, lane 2). PE migrated as a major polypeptide with M_r 33,000, and PAP migrated as doublet bands with approximate M_r 50,000 (Fig. 1, lanes 3 and 9, respectively). The doublet bands of PAP and the smear above the single bank of PE are best explained by the lack of a protein carrier effect, since neither was seen in samples containing identical amounts of PE and PAP incubated with laminin (Fig. 1, lanes 4 and 10, respectively). PE extensively degraded laminin A and B chains in a dose-dependent manner (Fig. 1, lanes 4 to 8) with the limit digest containing laminin fragments with approximate M_rs 60,000, 17,000, and 25,000 (lanes 4 and 5). PAP extensively degraded the A chain more than the B

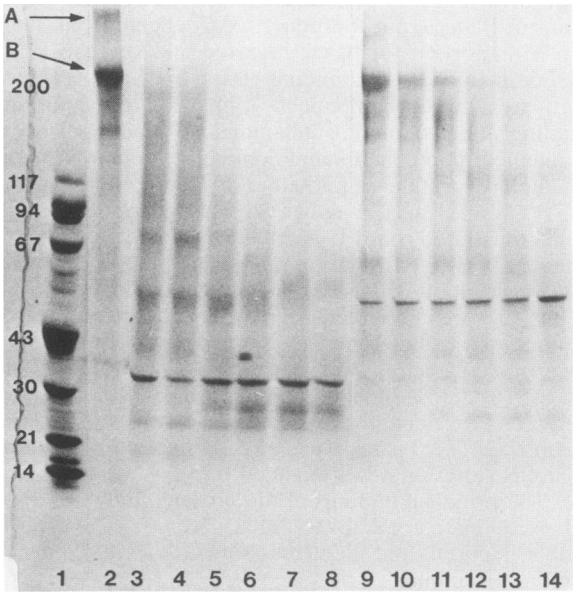

FIG. 3. Coomassie blue staining of SDS-polyacrylamide gradient gel electrophoresis run under reducing conditions after incubating constant amounts of laminin with PE or PAP for variable times at 37°C. In lane 1 are the globular protein molecular weight standards. In lane 2 is 30 μg of laminin. In lanes 3 through 8 are 30 μg of laminin incubated with 1 μg of PE for 5, 15, 60, 120, and 240 min, respectively. In lanes 9 through 14 are 30 μg of laminin incubated with 1 μg of PAP for 5, 15, 60, 120, and 240 min, respectively.

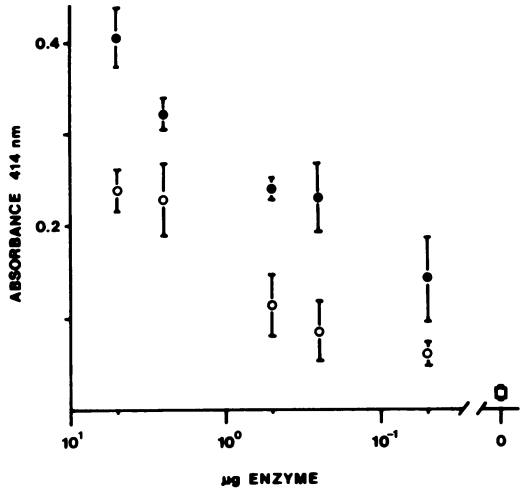

FIG. 4. Plot of data from a tissue digestion enzyme-linked immunosorbent assay conducted as described in Materials and Methods. Quantitatively more laminin was released from kidney sections treated for 60 min with PE (●) than from sections treated with PAP (○). In both cases, however, much more laminin was released than when sections were treated for 60 min with buffer alone (□). Error bars represent the standard errors of the mean from triplicate assays.

chains (Fig. 1, lanes 10 to 14), with limit digest cleavage fragments with approximate M_rs 125,000, 88,000, 75,000, 43,000, and 32,000.

The PE limit digest in unreduced gels revealed laminin fragments with approximate M_rs 155,000, 88,000, 70,000, and possibly 50,000 (Fig. 2, lanes 4 and 5), whereas PAP generated only two fragments with approximate M_rs 85,000 and 72,000 which were very faintly seen (Fig. 2, lanes 11 and 12). Interestingly, smaller fragments of both PE and PAP incubated alone without laminin were seen in the nonreduced gel (Fig. 2, lanes 3 and 10), respectively), but not after reduction (Fig. 1, lanes 3 and 10, respectively), indicating that these lower-molecular-weight forms were disulfide-bonded autolytic fragments.

In the time course experiment (Fig. 3), there was extensive degradation of laminin A and B chains by PE even after a 5-min incubation (lane 3), whereas PAP rapidly cleaved the A chain (lanes 9 and 10) and slowly cleaved the B chains (lanes 12, 13).

Enzymatic digestion of basement membrane laminin. To determine the effect of PE and PAP on authentic basement membranes, two tissue degradation assays were developed. In the first assay, cryostat kidney sections were incubated with variable amounts of either enzyme or buffer. After inactivation of enzymatic activity, the supernatant fluid was assayed for immunoreactive laminin by using an enzyme-linked immunosorbent assay. Quantitatively more laminin

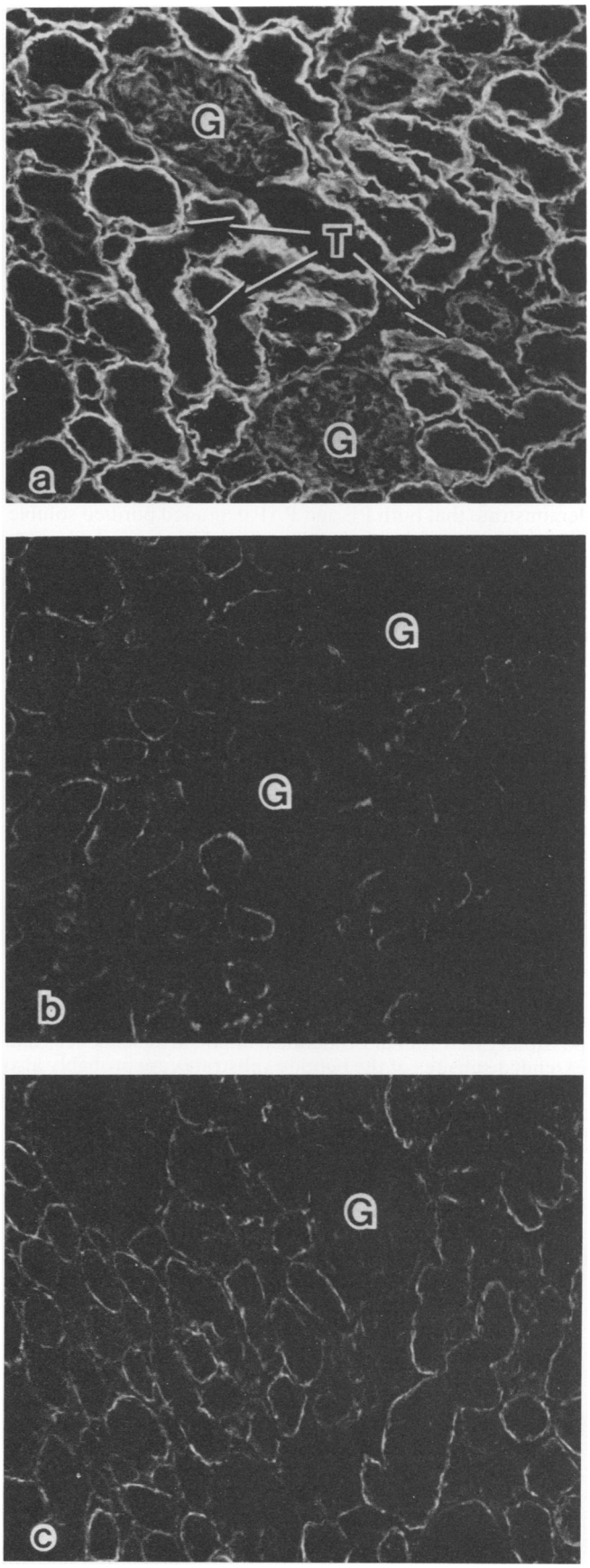

FIG. 5. Immunofluorescent photomicrographs of cryostat sections of unfixed rat kidney. Sections were treated for 2 min with buffer (a), PE (b), or PAP (c). Sections were then sequentially labeled with affinity-purified sheep anti-laminin IgG and fluorescein anti-sheep IgG. In sections treated with buffer only (a), there was intense fluorescence from basement membranes of glomeruli (G) and tubules (T). After treatment with PE (b) or PAP (c), there was a marked reduction in binding of anti-laminin IgG. Magnification, ×200.

was released from the kidney sections treated for 60 min with PE than from sections treated with PAP (Fig. 4). Sections treated with buffer alone released far less than those treated with either enzyme (Fig. 4). In the second assay, cryostat sections were flooded for 2 min with solutions of PE or PAP (0.25 μg/ml) or buffer. After washing, the sections were incubated sequentially with sheep anti-laminin IgG followed by fluorescein anti-sheep IgG and examined by epifluorescence. There was brilliant, linear fluorescence of tubular and glomerular basement membranes in sections treated with buffer alone (Fig. 5a). In contrast, there was marked reduction in basement membrane fluorescence in tubules and glomeruli treated with PE and PAP (Fig. 5b and c, respectively).

DISCUSSION

The availability of defined reagents allowed us to undertake a systematic study to investigate the possibility that purified and tissue-associated basement membrane laminin could be degraded by PE and PAP. The results clearly demonstrate that both PE and PAP degraded purified soluble laminin, as noted by the appearance of cleavage fragments after Coomassie blue staining. In addition, immunoreactive laminin was released from basement membranes after incubation with both enzymes, as detected with enzyme-linked immunosorbent and immunofluorescence assays. The release of laminin from basement membranes and the subsequent degradation as demonstrated in this paper suggest a role for PE and PAP in pathologic tissue resorption.

In the studies with soluble laminin, PE extensively degraded the A and B polypeptide chains in a dose-dependent and time-dependent manner, whereas PAP degraded the A chain more rapidly and extensively than the B chains. We conclude that the laminin A chain is very susceptible to proteolysis by both pseudomonas proteases. In contrast, the B chains contain a large disulfide-rich region that is relatively resistant to PAP proteolysis as seen in the short-timed dose-response experiments. However, with longer incubation times, the B chains were also eventually cleaved by PAP. The kinetic parameters and peptide bond cleavage sites of some simple synthetic peptides for both PE (23, 24) and PAP (23, 26) have been reported. This raises the possibility that similar or identical amino acid sequences and extended subsites of the peptide substrates are probably also found in the laminin chains, although the complete primary structure for laminin is not known.

It was possible that the results obtained in these studies with highly purified components may not accurately reflect the in vivo matrix degradation seen in infected tissues. Therefore, two independent tissue assays were devised to demonstrate release of laminin from tissues rich in basement membranes. The rapid release of immunoreactive laminin from the tissue sections and diminished immunofluorescence staining strongly suggest that both enzymes may function in pathologic tissue degradation.

Recent reports have shown that *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus sanguis*, but not several gram-negative bacteria (*Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Serratia marsecans*),bind preferentially to tissue culture wells coated with laminin, fibronectin, and type IV collagen (37). In addition, Lopes et al. have reported that specific binding of laminin to *S. aureus* (but not to *S. epidermidis*) occurs by a defined cell wall surface receptor (19). The relevance of these findings to *Pseudomonas* infectivity is currently unknown, but perhaps the adhesion and subsequent degradation of extracellular matrix components such as laminin promote the infectivity process.

Our studies reported in this paper on the apparent loss of laminin from kidney basement membranes are consistent with the histologic findings in other tissues infected with *P. aeruginosa* and in experimental tissue injury induced by inoculation of various tissues with pseudomonas proteases (9, 15, 17) or with *P. aeruginosa* (4, 8, 29, 31). How PE and PAP affect tissue invasion and hemorrhagic necrosis may be explained by protease-substate specificities of primarily PE but also PAP. Furthermore, laminin is the first extracellular matrix component reported to be degraded by PAP. Once the epithelial surface is denuded or damaged, colonization with *P. aeruginosa* occurs, followed by the extracellular release of proteases. PE may rapidly degrade laminin, elastin, and types III and IV collagens, which normally function as structural proteins to maintain tissue integrity. Proteolysis of these extracellular matrix proteins is an end stage process, since no reparative mechanism exists to restore chain length and function. If degradation exceeds synthesis of these macromolecules, epitelial and vascular integrity cannot be maintained, which ultimately leads to tissue necrosis. Our data would support the concept that PE and PAP are virulence factors.

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