Pseudomonas Elastase Acts as a Virulence Factor in Burned Hosts by Hageman Factor-Dependent Activation of the Host Kinin Cascade

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Purified Pseudomonas elastase injected subcutaneously into the skin of an Evans blue dye-injected (intravenously) guinea pig caused dye leakage similar to that observed when histamine or bradykinin was injected in the same animal. The histamine-induced dye leakage was ablated in antihistamine-treated guinea pigs, but elastase- and bradykinin-induced dye leakages were not. Local injections of specific inhibitors of the host Hageman factor-dependent bradykinin-generating pathway given immediately prior to elastase injection reduced dye leakage in a dose-related manner. Elastase-related dye release was enhanced when angiotensin-converting enzyme inhibitor, a substance which prevents host enzymes from breaking down bradykinin, was injected prior to elastase injection. We conclude that Pseudomonas elastase generates bradykinin in the infected host via a Hageman factor-dependent pathway.

Nosocomial infections by Pseudomonas aeruginosa continue to be a leading cause of morbidity and mortality among hospitalized patients with a variety of underlying diseases, such as cystic fibrosis, burns, cancer, and other immunosuppressive illnesses (4, 24). In addition, P. aeruginosa is an important cause of a variety of rapidly progressing and destructive ocular diseases (1, 29), and mortality due to Pseudomonas pneumonia has been reported to be as high as 50 to 80% (23, 27).

P. aeruginosa elaborates a large number of virulence-associated exoproteins, including exotoxin A, exoenzyme S, and the proteolytic enzymes elastase and alkaline protease (12), which may allow the P. aeruginosa organisms to cause this wide array of infections. Different virulence factors have been implicated in different infectious processes. Thus, exotoxin A and alkaline protease production, but not elastase elaboration, has been shown to be important in P. aeruginosa-associated eye infections (11, 21), whereas elaboration of exotoxin A, exoenzyme S, and elastase, but not alkaline protease, has been shown to be an important virulence-associated factor for P. aeruginosa strains causing respiratory infections (2, 32).

In the case of burns, P. aeruginosa strains must elaborate at least exotoxin A and proteases to cause infections (6, 8, 22, 25, 26). While the mean time to death of antitoxin-treated burned P. aeruginosa-infected mice was increased relative to that of untreated controls, no long-term survival was achieved (26), whereas antiprotease therapy with protease inhibitors led to significantly enhanced long-term survival (6, 8, 9, 18). Curiously, in some of the protease inhibitor treatment studies, enhanced survival was observed when protease inhibitors which had no activity against P. aeruginosa proteases but which were active against host proteases were used (9, 18). Why treatment with protease inhibitors which inhibited only host proteases should enhance survival in burned animals infected with P. aeruginosa was unclear. Results presented in this study provide insight into this phenomenon.

MATERIALS AND METHODS

Animals and experimental model. The animal model used was that of Kamata et al. (13). Briefly, 350- to 450-g male albino Hartley guinea pigs were used. The backs were shaved, and the animals were anesthetized, after which an injection of Evans blue dye, the dosage adjusted to 30 mg/kg, was made via the dorsal penile vein. Twenty minutes later solutions containing purified Pseudomonas elastase (5 µg in a 0.1-ml volume) were injected subcutaneously into the guinea pig skin. At 15 min postinjection, the guinea pigs were sacrificed, the dorsal skin was flayed, and permeability activity was evaluated both by visual assessment of blue color produced by extravascular dye leakage and by spectrophotometric quantification of dye extracted from each experimental skin site (see below). For some experiments, both histamine and bradykinin injections given subcutaneously (5 and 3 µg, respectively) were given to dye-injected guinea pigs, some of which were pretreated (90 min) with the antihistamine triprolidine hydrochloride (200 µg/kg) intravenously. The intensity of color and the extent of dye leakage in histamine- and bradykinin-injected sites were compared with those in sites injected only with elastase (serving as controls in the same animals). In other animals, additional substances (see below) were injected with elastase.

Vascular permeability-activating substances and inhibitors. Purified Pseudomonas elastase was purchased from Nagase Biochemicals, Ltd. (Kyoto, Japan), while Evans blue dye, histamine, bradykinin, soybean trypsin inhibitor (SBTI), angiotensin-converting enzyme (ACE) inhibitor, and tripolidine hydrochloride were all purchased from Sigma Chemical Co., St. Louis, Mo. Corn trypsin inhibitor (CTI), prepared as described by Lei and Reек (15), was kindly supplied by Gerald Reек, Kansas State University, Manhattan. To determine the effects of some of these substances on elastase-induced dye release, we injected SBTI, CTI, and ACE inhibitor in the amounts indicated on the figures into the skin of dye-injected animals together with elastase. For each substance injected, comparisons were made between dye release in a site injected with elastase plus inhibitor...
Pseudomonas elastase dye-injected intradermal substances control, blue dark the same control. associated a versus in treated (B) animals. extravascular dye leakage (data evoked elastase were pigs et al. 3346 HOLDER AND All tively. inflammation, showing necessary for the nontreated the animals. FIG. 1. Comparison of dye leakage lesions in the skin of Evans blue dye-injected guinea pigs following local injection of 5 µg of Pseudomonas elastase (sample 1), 3 µg of histamine (sample 2), or 10 µg of bradykinin (sample 3) in untreated (A) and antihistamine-treated (B) animals.

versus a site injected only with elastase (serving as a control in the animal).

Spectrophotometric quantitation of dye leakage. Evans blue dye associated with inflammatory skin lesions produced by intradermal injections of various vascular permeability-active substances or their inhibitors was extracted and quantified by the spectrophotometric method described by Udaka et al. (30). In each experiment the absorbance for the sample injected only with elastase was considered to be the 100% control, with the absorbance for the experimental samples in the same animal being calculated as a percentage of the control.

RESULTS AND DISCUSSION

Initial experiments in which elastase was injected into the skin of Evans blue dye-injected guinea pigs showed that elastase evoked a local inflammatory response observed as dark blue circumscribed lesions in the skin caused by extravascular dye leakage (data not shown). Heat-inactivated (100°C for 5 min) elastase injection did not cause inflammation, showing that biologically active enzyme was necessary for the reaction to occur. A possible explanation for the cause of this reaction was that elastase injection stimulated the host elaboration of the inflammatory response mediator histamine or bradykinin or both. Therefore, guinea pigs were injected with these substances as well as with elastase (Fig. 1). Panels A and B represent results obtained in nontreated and antihistamine-pretreated animals, respectively. All injected substances caused dye release lesions in the nontreated animals (Fig. 1A, samples 1 to 3). In the antihistamine-pretreated animals, dye release caused by histamine injection was ablated (Fig. 1B, sample 2), whereas antihistamine pretreatment had no effect on dye release caused by injection of either elastase or bradykinin (Fig. 1B, samples 1 and 3, respectively). Therefore, histamine was eliminated from consideration, and we concentrated our attention on bradykinin generation as the main candidate causing the observed dye leakage after elastase injection. Bradykinin is generated via a sequential cascading series of steps, starting with the activation of the Hageman factor (step 1), followed by conversion by activated Hageman factor of prekallikrein to kallikrein (step 2), which, in turn, converts kininogen to bradykinin (step 3) (14). In vivo, bradykinin is broken down into inactive peptides by ACE. Steps 2 and 3 can be specifically inhibited by CTI and SBTI, respectively (10, 16). Inhibition of these steps would be observed in our model as a diminution of dye leakage in the guinea pig skin as compared with dye leakage in control areas given the same initiating stimulus, in this case elastase injection. Conversely, inhibition of ACE by its inhibitor would appear as an intensification of dye leakage, since bradykinin would not be degraded to inactive peptide and would continue to accumulate in response to the initiating stimulus. In our experimental system, the specific inhibitors CTI, SBTI, and ACE inhibitor all produced the appropriate results, as outlined above (Fig. 2A to C). As compared with 100% dye leakage in elastase-injected control sites, 10 and 100 µg of CTI injected along with elastase reduced dye leakage to 68 and 58%, respectively (Fig. 2A, samples 2 and 3), of that in the control (Fig. 2A, sample 1), while 10 and 100 µg of SBTI injected along with elastase reduced dye leakage to 42 and 24%, respectively (Fig. 2B, samples 2 and 3), of that in the control (Fig. 2B, sample 1). Figure 2C, samples 2 and 3, shows enhanced dye release of 216 and 238% over that in the control (Fig. 2C, sample 1) when 5 and 50 µg, respectively, of ACE inhibitor were injected along with elastase. These results are consistent with the interpretation that elastase causes an inflammatory response through bradykinin generation via the activation of a Hageman factor-dependent pathway. These data confirm the recently pub-
lished results of Molla et al. (17), who showed that not only *Pseudomonas* elastase but also *Pseudomonas* alkaline protease and several other microbial proteases cause direct activation of the Hageman factor.

How this activity of *Pseudomonas* elastase relates specifically to the host-parasite interaction that constitutes a *P. aeruginosa* infection in burns cannot be understood fully without some consideration of the burned host in which the interaction occurs.

Minor thermal injury results in a local inflammatory response. In a major burn this local response becomes systemic, resulting initially in burn shock and subsequently in the postburn immunosuppression syndrome (3, 31). The data presented here suggest an additional, albeit indirect, means by which *P. aeruginosa* infections may cause immunological deficits via inflammatory response mediators, thereby making recovery from the burn plus infection less likely. The following presents our hypothetical concept of how this might occur.

The Hageman factor occupies a position at the apex of a number of interrelated protease-activated and protease-generating cascading pathways, the activation of which has some immunological consequences (3) (Fig. 3). Under normal circumstances these systems are kept in delicate balance, the activation of one or more systems when this is appropriate to the needs of the host being followed by inhibition by circulating plasma inhibitors when the needs of the host are met. After thermal injury of sufficient size, the kininogen-kinin, coagulation, fibrinolytic, and complement systems are activated (Fig. 3), and this activation is due, at least in part, to the Hageman factor (unpublished results). The generation of more proteases from these systems than the serum protease inhibitors can neutralize could account in part for the increase in total circulating proteolytic activity above that released from burned tissue per se (19). As a result of this undercontrolled proteolysis, the burned host becomes immunocompromised and subject to a wide variety of infections. However, despite all the bacterial threats to which burn patients are subjected, infections with *P. aeruginosa* cause the most death from sepsis in these patients; in one institution, 66% of all gram-negative septic deaths from 1964 to 1983 were caused by this organism (7). We postulate that one reason that burned hosts are particularly susceptible to *P. aeruginosa* infections is that this microorganism produces proteases which further activate the protease-generated immunosuppressive events (Fig. 3) which have already been initiated by the burn. The following experimental findings support this possibility: (i) burned animals are highly susceptible to *P. aeruginosa* infections, but 50% lethal doses of many other organisms do not vary much in burned versus nonburned animals (28), (ii) only elastase-producing *P. aeruginosa* strains are lethal in burned hosts (6, 8, 22, 26), and (iii) the survival of burned *P. aeruginosa*-infected mice is increased by treatment with a protease inhibitor (9, 18), an inhibitor which has no activity against *Pseudomonas* elastase but which both inhibits proteases in the host coagulation and fibrinolytic systems and neutralizes host neutrophil elastase (5, 20). Thus, *P. aeruginosa* infections in burns represent an unusual host-parasite interaction in which the initial posttrauma immunological derangements in the burned host are exacerbated by colonization by *P. aeruginosa*, making the host even more immunologically defenseless (Fig. 3).

In conclusion, we have presented evidence that *Pseudomonas* elastase activates host bradykinin via activation of the Hageman factor. The consequences of this are to activate "down-line" cascade systems and generate protease activity in the process, putting a further burden on the control function of the circulating protease inhibitors. This function, already heavily taxed by thermal injury, becomes...
exacerbated by infection with elastase-producing *P. aeruginosa*. The combination of thermal injury plus infection by elastase-producing *P. aeruginosa* creates in the host a circumstance in which the negative immunological events listed in Fig. 3 occur to a greater extent than those induced by the burn itself. We believe that it is this activity of elastase which allows *P. aeruginosa* infections in burn patients to proceed to a lethal outcome with much greater frequency than infections with other bacteria. While elastase may serve as a virulence factor for *Pseudomonas* infections in a variety of patient populations (12), we believe that Hageman factor-dependent kinin generation as a mechanism of virulence applies only in burn or perhaps other trauma circumstances, in which this activity of elastase becomes additive to the cascade activation already caused by the underlying condition in the host.

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