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Effect of Synthetic Protease Inhibitors of the Amidine Type on Cell Injury by *Rickettsia rickettsii*

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To evaluate the importance of proteolytic activity in the pathogenesis of cell injury by *Rickettsia rickettsii*, a series of four aromatic amidine inhibitors of trypsin-like proteases were introduced into the plaque model. The compounds were shown to be active toward plaque reduction with their order of effectiveness parallel to their antitrypsin activity. One of the compounds, bis(5-amidino-2-benzimidazolyl)-methane, at a concentration of 10^{-5} M demonstrated complete inhibition of plaque formation on day 6. Bis(5-amidino-2-benzimidazolyl)methane at the same concentration reduced cell injury even when added to the system after 72 h of rickettsial infection. The reduction in morbidity in guinea pigs experimentally infected with *R. rickettsii* and treated with bis(5-amidino-2-benzimidazolyl)methane as compared with morbidity in infected, untreated animals, comprised delay in the onset of fever and slightly fewer febrile animals. Because bis(5-amidino-2-benzimidazolyl)methane had no effect on phospholipase A_2, the enzyme activity associated with penetration-induced cell injury, it is likely that a trypsin-like protease also plays an essential role either in the physiology of *R. rickettsii* or as its pathogenic mechanism.

Virions of several different genera require exposure to proteolytic enzymes to achieve full expression of their biological properties (7). As a corollary to this fact, protease inhibitors can be expected to have potential antiviral activity. This was substantiated by our recent discovery of the ability of synthetic low-molecular-weight inhibitors of trypsin-like proteases to block respiratory syncytial virus-induced cytopathology (2–4, 10). Although the site of action of these agents has not yet been determined, we presented strong evidence that their antiviral effect may be linked to their antiproteolytic properties. The most potent of the inhibitory agents, bis(5-amidino-2-benzimidazolyl)methane (BABIM), was shown to exert the following effects: (i) delay of penetration of virus into cells, (ii) blockage of virus-induced cell fusion, (iii) reduction of multiple-cycle yields of virus, and (iv) reduction in pathology and virus yield in experimentally infected animals.

Similar to viral diseases, rickettsial infection of cells involves a penetration step. This event is known to be enzyme mediated, but thus far only phospholipase A_2, and not a protease, has been shown to participate in the process (15, 23). Our experience with respiratory syncytial virus suggested application of the amidine inhibitors to the rickettsial system in search of evidence of proteolysis in the pathogenetic sequence. Such evidence was readily found in a study of the rickettsial plaque assay, and the results are the subject of this communication.

MATERIALS AND METHODS

Rickettsiae. Stocks of *R. rickettsii* (Sheila Smith strain) were cultivated by inoculation of the yolk sac of 5-day-old specific pathogen-free embryonated hen eggs (SPAFAS, Norwich, Conn.) with plaque-purified organisms provided by Charles L. Wisseman, Jr. (University of Maryland, Baltimore). Inoculated eggs were incubated at 35°C, and their yolk sacs were harvested 5 days after inoculation, 24 to 48 h after the death of the chick embryos. Yolk sacs containing rickettsiae were homogenized in a Waring blender, diluted in sucrose phosphate glutamate (0.218 M sucrose, 0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4, 0.0049 M glutamate, pH 7.0) (1) to a 1% suspension, and stored frozen at −70°C in 1-ml samples. Samples were titrated by plaque assay in chick embryo cell culture and found to contain 7×10^5 PFU/ml.

Plaque model. Flasks (Corning Glass Works, Corning, N.Y.) with 25-cm^2 monolayers of E6 clone Vero (African green monkey kidney) cells were inoculated with 0.1 ml of a suspension of *R. rickettsii* diluted 10^{-3} in brain heart infusion broth. The inoculum was adsorbed for 30 min before the addition of 5 ml of minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) containing 5% fetal bovine serum (Flow Laboratories, Inc., McLean, Va.), 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, 2 mM L-glutamine, ca. 0.075% $NaHCO_3$ to final pH 7.3, 0.5% agarose (Sea Kem, FMC Corp., Marine Colloids Div., Rockland, Maine), and different concentrations of protease inhibitors. Flasks were incubated at 35°C for 4 days at which time 5 ml of an identical second overlay medium containing 0.01% neutral red was added. After further incubation, plaques were counted on various combinations of days 5, 6, and 7 (14, 16, 20–22).

Protease inhibitors. Inhibitors of trypsin-like proteases used in these experiments were BABIM, 1,2-bis(5-amidino-2-benzimidazolyl)ethane, 1,5-bis(5-amidino-2-benzimidazolyl)pentane, and 5-amidinoindole. They were synthesized as previously reported (5, 9).

Experimental design. Each of these aromatic amidines was incorporated into both the first and second overlay media of three flasks at concentrations of 10^{-4}, 10^{-5}, and 10^{-6} M, and plaque counts were compared with those of monolayers inoculated with the same rickettsial suspension and overlaid with medium containing no protease inhibitors. Control flasks included uninoculated, untreated monolayers and also uninfected monolayers treated with a 10^{-4} M concentration of the aromatic amidines for evaluation of toxicity. The mean plaque count was calculated for each concentration of

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TABLE 1. Effect of inhibitors of trypsin-like proteases on formation of plaques with *Rickettsia rickettsii*

Compound	Concn (M)	Effect of inhibitors on day[a]: 5 Plaques	5 Size	6 Plaques	6 Size	7 Plaques	7 Size	Trypsin K_i (μM)
Bis(5-amidino-2-benzimidazolyl)-methane	10^{-4}	—	—	0 ± 0	O	0 ± 0	O	0.017
	10^{-5}	—	—	0 ± 0	O	15.0 ± 3.5	S	
	10^{-6}	—	—	46.0 ± 1.5	S	52.0 ± 1.2	S	
	None	—	—	70.0 ± 5.0	N	68.5 ± 5.5	N	
1,2-Bis(5-amidino-2-benzinidazolyl)-ethane	10^{-4}	Toxic		Toxic		Toxic		4.68
	10^{-5}	21.7 ± 3.7	N	23.7 ± 3.8	N	23.0 ± 5.0	N	
	10^{-6}	22.3 ± 5.8	N	29.0 ± 7.6	N	31.7 ± 9.4	N	
	None	32.0 ± 3.0	N	37.3 ± 3.2	N	38.0 ± 2.6	N	
1,5-Bis(amidino-2-benzimidozolyl)-pentane	10^{-4}	—	—	0 ± 0	O	—	—	9.46
	10^{-5}	—	—	60.7 ± 5.2	S	—	—	
	10^{-6}	—	—	67.3 ± 2.4	N	—	—	
	None	—	—	71.0 ± 0.8	N	—	—	
5-Amidinoindole	10^{-4}	34.7 ± 2.0	N	51.7 ± 0.9	N	—	—	29.1
	10^{-5}	42.7 ± 1.2	N	56.7 ± 1.7	N	—	—	
	10^{-6}	Contam.		Contam.		—	—	
	None	39.5 ± 5.5	N	58.0 ± 6.0	N	—	—	

[a] Plaques are measured by mean number of plaques per flask ± standard error of the mean and by size: —, not examined; O, no plaques; S, small plaques; N, normal-size plaques; Contam., culture contaminated.

each amidine and was compared with the count of the *R. rickettsii*-infected flasks containing no amidine.

In a second experiment, the first overlay after inoculation of the monolayer with *R. rickettsii* contained no aromatic amidines. On day 3 (72 h after inoculation of rickettsiae), a second overlay was added to each flask. The second overlay contained BABIM at concentrations of 2×10^{-4}, 2×10^{-5}, and 2×10^{-6} M to achieve final protease inhibitor concentrations of 10^{-4}, 10^{-5}, and 10^{-6} M in the combined overlay medium.

Guinea pig experiment. Twenty-five adult (400- to 600-g) male guinea pigs (Hartley strain) were divided as follows. Ten animals were inoculated intraperitoneally with 330 50% tissue culture infective doses (ca. 38 50% guinea pig infectious doses) of *R. rickettsii* (Sheila Smith strain) and treated with BABIM at a dose of 15 mg/kg per day given daily by the intraperitoneal route beginning 30 min after rickettsial inoculation and continuing for 9 days; ten animals were inoculated intraperitoneally with 330 50% tissue culture infective doses of *R. rickettsii* and given no treatment, and five uninfected animals were given 15 mg of BABIM per kg daily via the intraperitoneal route for 9 days. Animals were examined daily, and rectal temperatures were measured with a battery-operated thermometer with a flexible probe (Telethermometer; Yellow Springs Instrument Co., Yellow Springs, Ohio).

Phospholipase A_2 assay. The procedure used was that described by Vigo et al. (12), in which hydrolysis of phospholipid-containing liposomes is followed spectrophotometrically at 340 nm. The assay mixtures of 1 ml of 0.1 M Tris-hydrochloride buffer (pH 7.2) contained 0.5 mg of dipalmitoyl-lecithin (as liposomes), 1 mM $CaCl_2$, and 10 U of phospholipase A_2 from *Naja naja* venom (Sigma Chemical Co., St. Louis, Mo.). The reaction was terminated by the addition of 1 ml of methanol containing 15 mM EDTA.

RESULTS

The effects of inhibitors of trypsin-like proteases on plaque count and size are presented in Table 1. There was a close correlation between the reduction in plaque count and the inhibition constants (K_i values) for trypsin (2). The most marked plaque reduction was observed with the most effective inhibitor of trypsin activity, BABIM, with no plaques being observed on day 6 after inoculation in the presence of 10^{-5} M of the amidine. Measurable plaque reduction was also observed at a concentration of 10^{-6} M BABIM, and the plaques present were smaller than untreated plaques. The least active inhibitor of trypsin, 5-amidinoindole, which has a K_i of 29.1 μM for trypsin (5), showed minimal effects on *R. rickettsii* plaque count and size. The monolayers treated with 1,5-bis(5-amidine-2-benzimidazolyl)pentane, having an intermediate K_i (9), showed evidence for reduction in cell injury caused by *R. rickettsii* as measured by plaque count and size. However, the effect was less than that of BABIM. At concentrations of 10^{-4} M and less, none of the aromatic amidines except 1,2-bis(5-amidino-2-benzimidazolyl)ethane caused cytotoxic effect on the Vero cells.

To determine whether delayed exposure to BABIM would still influence the cytopathic events in the monolayers, a second series of experiments was carried out. Here, the Vero cell culture was inoculated with *R. rickettsii*, and the establishment of infected foci was allowed to proceed normally by feeding with an initial overlay free of BABIM. On day 3 after inoculation, second overlays containing various concentrations of the inhibitor were added, and the resulting effects on the monolayer on day 6 and 7 were recorded (Table 2). As can be seen, there was complete suppression of plaque formation at a BABIM concentration of 10^{-4} M. At a concentration of 10^{-5} M, there was still a notable reduction in plaque count. However, the effect was less pronounced than it was when BABIM had been present in the medium immediately after the inoculation (Table 1).

BABIM was somewhat toxic for guinea pigs, causing a transient fever (≥40°C) early in the course of treatment (4 of 5 animals on day 3 and 2 of 5 animals on day 4) (Table 3). A similar transient fever was observed early in the course of BABIM treatment of animals infected with *R. rickettsii*. However, the temperatures of all guinea pigs returned to base line before day 5. Onset of fever was delayed; 5 of 10

TABLE 2. Effect of treating *Rickettsia rickettsii* plaque model with BABIM on day 3 after inoculation

BABIM concn (M)	Effect on day[a]: 6		7	
	Plaques	Size	Plaques	Size
10^{-4}	0 ± 0	O	0 ± 0	O
10^{-5}	31.3 ± 5.4	N	50.0 ± 13.0	S
10^{-6}	67.7 ± 9.0	N	82.7 ± 9.5	N
None	76.3 ± 3.8	N	82.0 ± 3.1	N

[a] Plaques are measured by mean number of plaques per flask ± standard error of the mean and by size: O, no plaques; N, normal plaque size; S, small plaques.

untreated animals had fever on day 5 as compared with no febrile animals in the BABIM-treated group. On day 6, 8 of 10 untreated animals were febrile as compared with only 2 of 10 BABIM-treated animals. Four febrile animals in each group died during the course of the rickettsial disease.

BABIM was found to have no effect on the hydrolysis of dipalmitoyl-lecithin liposomes by phospholipase A_2 from *Naja naja* venom.

DISCUSSION

The order of effectiveness of aromatic amidines in reducing plaque formation by *R. rickettsii* follows their order of effectiveness in inhibiting trypsin and several other trypsin-like proteases and corresponds to their order of effectiveness in blocking cell fusion by respiratory syncytial virus in vivo (2, 5, 10). This parallelism of activities at low concentrations argues strongly for the antirickettsial effect resulting from the action of compounds as protease inhibitors on a trypsin-like enzyme. However, it was also necessary to consider the possibility that amidines might act by inhibition of phospholipase A_2, especially since a cell penetration phospholipase appears to be an important rickettsial pathogenic mechanism and a protease inhibitor of a different structure had previously been shown to suppress phospholipase A_2 activity (8). We showed this possibility to be unlikely by demonstrating that BABIM at a concentration of 10^{-4} M has no effect on phospholipase A_2 activity (from *Naja naja* venom). Of course, the linkage of a trypsin-like protease in a chain of enzymes that includes phospholipase A_2 cannot be excluded (11). In fact, trypsin activation is necessary for all mammalian pancreatic phospholipases A_2 (13). Some of the other possible mechanisms by which a trypsin-like protease might fit into the scheme of rickettsial injury to cells, besides proteolytic activation of the penetration mechanism-associated phospholipase, include direct proteolytic attack on the host cell membrane either during entrance into the cell or on release from the cell or an essential intracellular catabolic function.

The reduction of plaque counts by the addition of BABIM 72 h after the establishment of rickettsia-infected foci documents that the protease inhibitors are not merely preventing initial rickettsial infection and suggests that protease inhibitors may be blocking a rickettsial function essential to expression of the pathogenic mechanism. The hypothesis that a protease-associated pathogenic mechanism is blocked by BABIM is also supported by the delay of plaque formation by a 10^{-5} M concentration of BABIM with delayed appearance of several small plaques on day 7. Rickettsiae survived the BABIM treatment and caused formation of a few plaques, presumably after protease activity overcame the protease inhibitory activity. Because fatal cases of Rocky Mountain spotted fever are often diagnosed and treated too late in the course of disease for rickettsiostatic antimicrobial agents to prevent the demise of the patients (6, 17–19), additional inhibition of rickettsial injury to the host would benefit such critically ill patients. Consideration of the possible use of inhibitors of trypsin-like proteases for the treatment of rickettsial diseases would require investigation of these drugs in other animal models, further study of the mechanism of action of protease inhibitors on the rickettsia-host cell interaction, and information on the toxicity of these compounds in humans, especially on the inflammatory and coagulation mechanisms: complement, kallikrein, coagulation, and fibrinolysis.

Data on the toxicity of BABIM include the acute 50% lethal dose for cotton rats of 136 mg of BABIM per kg and a dosage for cotton rats of 30 mg of BABIM per kg daily for 7 days without ill effects. Current pharmacological studies of

TABLE 3. Effect of BABIM on experimental infection of guinea pigs with *Rickettsia rickettsii*

Day	No.[a] of: Rickettsia-infected guinea pigs: BABIM treated			Rickettsia-infected guinea pigs: No treatment			Uninfected guinea pigs: BABIM treated		
	Afebrile	Febrile	Dead	Afebrile	Febrile	Dead	Afebrile	Febrile	Dead
1	10	0	0	10	0	0	5	0	0
2	6	4 (40.1)	0	10	0	0	5	0	0
3	2	8 (40.2)	0	10	0	0	1	4 (40.1)	0
4	6	4 (40.1)	0	10	0	0	3	2 (40.1)	0
5	10	0	0	5	5 (40.6)	0	5	0	0
6	8	2 (40.8)	0	2	8 (40.7)	0	5	0	0
7	6	4 (40.7)	0	2	8 (40.7)	0	5	0	0
8	5	4 (40.7)	1	2	8 (40.7)	0	5	0	0
9	4	5 (40.7)	1	3	7 (40.8)	0	5	0	0
10	6	3 (40.7)	1	2	7 (40.8)	1	5	0	0
11	4	3 (40.5)	3	4	5 (40.7)	1	5	0	0
12	6	1 (40.8)	3	5	3 (40.6)	2	5	0	0
13	6	1 (40.4)	3	4	3 (40.4)	3	5	0	0
14	6	1 (40.4)	3	4	3 (40.3)	3	5	0	0
Final	6	0	4	6	0	4	5	0	0

[a] The values in parentheses are the mean temperatures of febrile guinea pigs.

rats that received a dose of 20 mg of BABIM per kg daily and of mice that received a dose of 20 mg of BABIM per kg daily have not detected any toxicity. These observations of protease inhibitors blocking rickettsial injury to cells offer interesting new hypotheses for studying rickettsial pathogenesis. At this point, they should be used as tools for elucidating rickettsial physiology and pathogenic mechanisms.

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