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ALLANTOIC FLUID PROTEASE ACTIVITY DURING INFLUENZA VIRUS INFECTION

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Summary. — Neutral protease activity of allantoic fluid from embryonated chicken eggs was quantified during the course of influenza virus infection. Antigenic subtypes of influenza A viruses selected for study were H1N1 strains PR/8/34, Brazil/8/78, FM/1/47, the H3N2 strain Bangkok/1/80 and the H5N9 Turkey/ /Ontario/66 as well as the Sendai strain of parainfluenza type 1 virus. Three different types of profiles of allantoic fluid proteases could be readily distinguished after infection of eggs with various virus strains. In all profiles, periodic peaks of protease activity always preceded the partial shut down of protamine cleaving proteases which paralleled the production of near maximum titers of infectious virus. To determine the mechanism involved in this reduction of proteolytic activity, infectious allantoic fluids were analysed for the presence of protease inhibitors. Acid heat treated 48 hour virus-infected allantoic fluids of different influenza strains could inhibit the activities of subtilisin and allantoic fluid proteolytic enzymes.

Key words: influenza viruses; allantoic fluid; protease profiles; inhibitors

Introduction

The availability of appropriate host proteases and the susceptibility of the hemagglutinin (HA) glycoprotein to proteolytic cleavage are important for determining infectivity of the influenza viruses (Rott, 1982). Cleavage of the HA is highly specific. While proteolytic enzymes with different specificities can cleave the hemagglutinin, activation of virus infectivity results only after cleavage with trypsin or trypsin-like enzymes (Klenk *et al.*, 1977). There is evidence for the involvement of allantoic fluid proteases in cleaving the virus hemagglutinin. According to Maeda *et al.* (1981), in egg grown influenza virus, posttranslation processing of the HA is performed by proteases in host cells or chorioallantoic fluid. Also Zhirnov (1982) has found that supplementing the growth medium of influenza virus-infected chick embryo fibroblasts with 10 % allantoic fluid resulted in both a higher percentage of cleaved HA molecules and an increased infectivity titer. Plasmin, present in the allantoic fluid believed to be responsible for en-

hancing the HA cleavage. Similar findings were noted by Homma and Ouchi (1973) with the Sendai parainfluenza virus. They showed that biologically inactive Sendai virus, when incubated with allantoic fluid was converted to the active form. Previous fluorometric studies on quantification and partial characterization of allantoic fluid proteaes have confirmed the presence of proteolyic enzymes with the trypsin-like specificities necessary for hemagglutinin cleavage (Ewasyshyn and Sabina, 1983).

In the present investigation, neutral protease activity in the allantoic fluid of embryonated eggs was quantified throughout the course of influenza virus replication. Protease profiles of several different human avian viruses could be compared. In addition, infectious allantoic fluids were analysed for the presence of protease inhibitors responsible for the observed reduction in fluid protease activity.

Materials and Methods

Viruses. Influenza strains A/PR/8/34, A/Brazil/8/78, A/FM/1/47 (H1N1 viruses), A/Bangkok/1/80 (H3N2) and parainfluenza type 1 Sendai virus were provided by D. A. McLeod, Laboratory Centre for Disease Control, Ottawa, Canada. The influenza A/Turkey/Ontario/7732/66 (H5N9) avian virus was obtained from G. Lang, Ontario Veterinary College, Guelph, Canada. All strains were grown for 48 hr at 35 °C in 9-day-old embryonated chicken eggs inoculated with 25 to 40 tissue culture 50% infectious doses ($TCID_{50}$) of the virus. The methods for concentrating viruses from clarified allantoic fluids and virus purification have been described previously (Sabina *et al.*, 1981; Ewasyshyn and Sabina, 1983). The virus pellets were resuspended in phosphate-buffered saline (PBS) and stored at −60 °C.

Virus assays. Viruses were assayed for infectivity by a modification of the microtiter system adapted from Moore (1977). The haemagglutinating activity was determined with rooster red blood cells by the method of Palmer *et al.* (1975).

Neutral protease assay. Protease activity was fluorometrically determined by the method previously outlined (Ewasyshyn and Sabina, 1983).

Measurement of protease inhibition by allantoic fluid. The presence of protease inhibitor(s) in allantoic fluid was determined by mixing aliquots of various test fluids with proteases of known specific activity. For these experiments, three sets of reaction tubes were used. The first set (I) contained a reaction mixture (100 μl) made up of equal volumes of test inhibitor solution and enzyme preparation. The second (II) and third (III) sets representing control tubes consisted of inhibitor solution (50 μl) and enzyme preparation (50 μl), respectively. All tubes were preincubated for 1 hr at 35 °C before the addition of 100 μl of 1% herring protamine sulphate as substrate. These sets were then incubated for an additional 5 hr. Just prior to fluorometrical assaying for protease activity, the protein concentration in the control tubes was adjusted by adding 50 μl of the enzyme preparation to the second set and 50 μl of inhibitor solution to the third set. To account for background fluorescence contributed by free amino groups present in the enzyme, inhibitor or substrate solution, each preparation was preincubated separately at 35 °C for the entire length of the experiment. Fifty μl of enzyme and inhibitor were mixed with 100 μl of substrate and 50 μl portions were immediately removed for determination of relative fluorescence. Per cent protease inhibition was calculated according to the formula

$$\frac{(B + C) - A}{B + C} \times 100$$

where A, B, and C represent the relative fluorescence of series I, II and III tubes, respectively.

Chemicals. Sucrose (density gradient grade) was purchased from Schwarz/Mann, Orangeburg, N. Y. and acetone (spectrophotometric grade) from J. T. Baker Chemical Co., Phillipsburg, N. J. All other chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri.

Results

Proteolytic activity of egg allantoic fluids were monitored during the course of replication of influenza H1N1 strains PR, FM and Brazil (Fig. 1), to investigate the effect of virus multiplication on fluid protease levels.

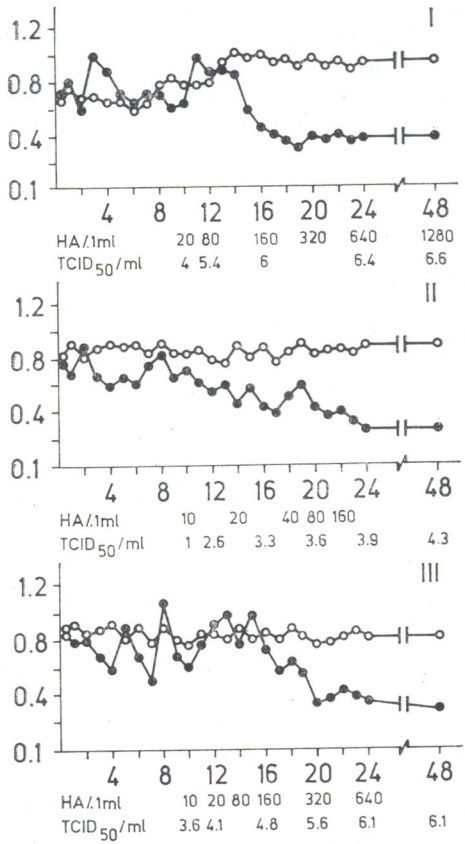

Fig. 1.

Allantoic fluid neutral protease activity during replication of different H1N1 influenza strains

Embryonated eggs were allantoically inoculated with about 40 $TCID_{50}$ of PR (I), Brazil (II), and FM (III) viruses. At hourly intervals for the first 24 hr and at 48 hr - p. i. allantoic fluid was harvested, clarified and assayed for protease, hemagglutinin and infectivity levels. Mean values for protease activity were derived from duplicate determinations on allantoic fluid samples pooled from 4 eggs. Only changes in hemagglutinin and infectivity titers are reported. Control eggs with tryptose phosphate broth as inoculum were similarly analyzed. Symbols: (○——○) control fluid protease activity; (●——●) virus-infected fluid activity.

Abscissa: hr p.i.; HA — hemagglutination units per 0.1 ml; infectivity in $\log_{10}$ $TCID_{50}$/ml; ordinate: relative fluorescence/μg protein.

The early peaks of protease activity detected in PR virus-infected fluid (Fig. 1-I) during the first 14 hr post-infection (p. i.) were approximately equal to or higher than control enzyme levels. Thereafter, there was a gradual decline in the level of enzymes capable of cleaving protamine. This decrease in enzyme activity paralleled the increase in HA and infectivity titers. In contrast, protease activity in mock-infected eggs remained high through the 48 hr test period. In three experiments with PR virus, the coefficient of variation for protease samples harvested hourly from control and virus-infected eggs was 9.5 and 9.6 %, respectively (data not shown). Thus, only single experiments were done to generate protease profiles of

other viruses. Differences were evident in the protease profiles obtained with the Brazil and FM strains. The peaks of protease activity during replication of the Brazil strain occurred mainly below control values when compared with the PR strain (Figs. 1-I and-II). As with the PR strain, the production of detectable HA and infectious virus was accompanied by steadily decreasing protease levels. By contrast, Fig. 1-III shows that replication of the FM strain led to pronounced protease peaks extending above control enzyme levels until 16 hr p. i. Maximum infectious titers were paralleled by the partial shut down of enzyme activity. These results demonstrate the ability of various H1N1 strains to induce characteristic patterns of periodic peaks of protease activity in allantoic fluid during virus replication.

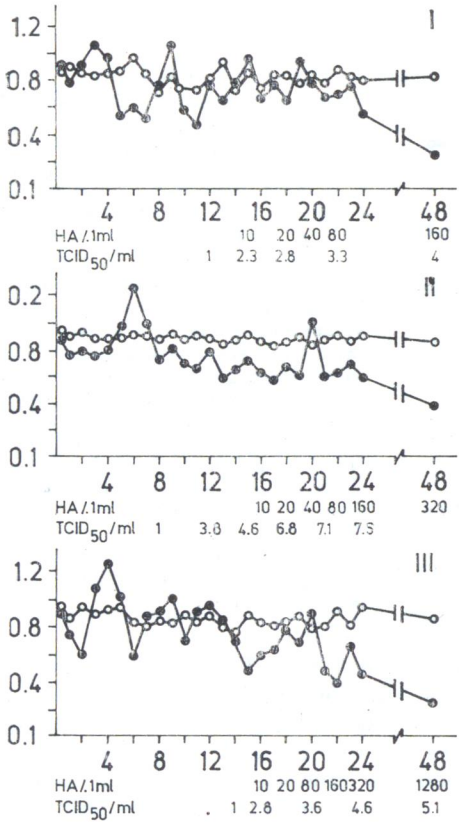

Fig. 2.
Allantoic fluid neutral protease activity during replication of Bangkok (I), Turkey/Ontario (II) and Sendai (III) viruses Experimental protocol as for Fig. 1 except an infective dose of approximately 25 $TCID_{50}$ of Turkey/Ontario virus per egg was used.
Abscissa: hr p.i.; HA units per 0.1 ml; infectivity in $\log_{10}$ $TCID_{50}$/ml; ordinate: relative fluorescence/μg protein

When viruses with properties that differ from influenza H1N1 strains were tested for their ability to induce characteristic changes in allantoic fluid protease activity, striking similarities in protease profiles of Bangkok

and Sendai strains were found. Replication of Bangkok (Fig. 2-I) and Sendai strains (Fig. 2-III) over 20 hr induced peaks of activity which extended mostly above that of mock-infected eggs. Also, despite the production of HA and infectious virus, enzyme levels were not continuously depressed until 24 hr p. i. These results resemble those shown for the FM strain (Fig. 1-III). In contrast, a much earlier shut down of protease activity at about 8 hr p. i. was observed in allantoic fluid of eggs infected with the Ontario/ /Turkey strain (Fig. 2-II). The protease profile of the Ontario/Turkey strain closely resembled that of the H1N1 Brazil strain (Fig. 1-II). Both viruses induced a characteristic progressive decrease in protease activity beginning at about 8 hr p. i. As in the case of H1N1 strains, the production of maximum levels of infectious virus at 48 hr was accompanied by a marked decrease in the level of neutral proteolytic enzymes with Bangkok, Ontario/Turkey and Sendai strains.

To eliminate the possibility that the observed sharp periodic oscillations of protease activity in profiles of virus-infected allantoic fluid was not the result of contamination of the inoculum with egg constituents, the PR strain was extensively purified by sucrose density gradient centrifugation. When purified or crude virus inoculum was used to infect eggs, essentially no differences were detectable in the enzyme profiles of infected allantoic fluids. It therefore seems improbable that the observed protease activity was induced by an egg fluid contaminant .The ability of noninfectious virus to influence proteolytic activity was studied. Comparison of the patterns of protease activity induced in eggs inoculated with either tryptose phosphate

Table 1. Per cent inhibition of initial peak of protease activity by 24 and 48 hr virus infected allantoic fluids

Virus[a]	Time of initial protease peak (hr)	Nontreated		Acid-heat treated[b]	
		24 hr*	48 hr*	24 hr*	48 hr*
PR	3[c]	0	27[c]	25[c]	59[c]
Brazil	2	19	20	22	40
FM	5	16	8	23	50
Bangkok	3	16	22	40	40
Turkey	6	23	ND[d]	33	ND
Sendai	4	7	13	25	25
Control	0	0	0	0	0

[a] Eggs were allantoically inoculated with approximately 25 $TCID_{50}$ of Turkey virus or 40 $TCID_{50}$ of the other viruses.

[b] Fluid was subjected to heating at 85 °C at pH 1 for 10 min and subsequently readjusted to pH 7.5 for analysis.

[c] ND, not determined due to hemolyzed red blood cells in the fluid.

[d] per cent inhibition.

* Twenty-four and 48 hr virus infected fluids were preincubated with allantoic fluid from the initial protease peak for 1 hr prior to the addition of substrate.

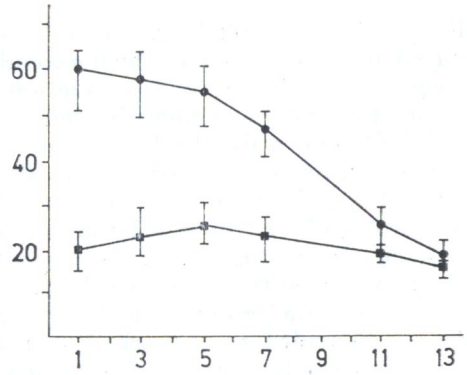

Fig. 3.
Effects of heat treatment and pH change on the inhibitory activity of 48 hr PR virus-infected allantoic fluid
Forty-eight hour PR infected allantoic fluid was treated at various pH values in the presence (●) or absence (■) of heating at 85 °C for 10 min. After readjustment of fluid to pH 7.5, treated fluid was tested for its ability to inhibit protease activity in 3 hr postinfection allantoic fluid. Inhibition of such proteases by heated-acidic 48 hr control uninfected allantoic fluid was not observed.

alone, stock PR virus or heat-inactivated PR virus (60 °C for 2hr), indicated that the profile of heat-inactivated virus was almost identical to that of mock--infected eggs. Thus, heat treatment abolished the viral property responsible for the irregular oscillations and subsequent depression of protease activity.

Attempts were made to elucidate the mechanism involved in the partial shut down of protease activity by highly infectious viral fluids. Such viral fluids were analyzed for the possible presence of protease inhibitors that may be involved in lowering enzyme activity. Using the PR strain, mixing experiments were done to determine whether 48 hr PR infectious fluid could reduce the initial protease peak obtained 3 hr p. i. Enzyme activity of the 3 hr sample was reduced only 30 % when preincubated with 48 hr infectious fluid for 1 to 3 hr prior to the addition of substrate. Because of the inability of the fluid to effectively block the activity associated with the initial protease peak, an attempt to maximize the inhibiting capacity of the fluid by the method of Lenney (1980) was examined. To selectively destroy proteases in 48 hr virus allantoic fluids which enhances the detection of thermostable protease inhibitors, fluids were heated at various pH values. These fluids, cooled and adjusted back to pH 7.5 were then mixed in equal volumes with 3 hr virus fluid and preincubated for 1 hr before the addition of substrate. Fig. 3 illustrates the inhibitory activity of 48 hr PR virus fluid. Protease activity of the 3 hr sample was maximally inhibited when 48 hr virus fluid at pH 1 was heated 10 min at 85 °C. Control uninfected 48 hr allantoic fluid treated in a similar manner was unable to block the 3 hr activity. The degree of protease inhibition progressively decreased under more alkaline conditions. In the absence of heating, the 48 hr virus fluid with the pH lowered to 1 resulted in only a 20 % reduction in 3 hr protease activity. Heating increased the per cent inhibition at least 3-fold. Apparently heat treatment of 48 hr PR allantoic fluid at the appropriate pH is necessary to demonstrate the inhibitory capability of the fluid.

Heat-treated acidic 24 and 48 hr allantoic fluids of other viruses were tested for their ability to reduce the initial peak of protease activity. Table

Table 2. Per cent inhibition of different proteases by 48 hr acid-heat treated allantoic fluids[a]

Protease	Conc. of protease (mg/ml)	Virus[b] PR	Brazil	FM	Bangkok	Turkey[c]	Sendai	Control
		% Inhibition[d]						
Trypsin	5×10^{-5}	0	0	0	0	0	0	0
Chymotrypsin	1×10^{-2}	0	0	0	0	0	0	0
Subtilisin	5×10^{-4}	73	77	100	100	90	67	0
Streptomyces griseus protease	5×10^{-4}	23	0	0	0	0	0	0
48 hr control allantoic fluid	?	50	50	54	50	40	57	11
48 hr control amniotic fluid	?	0	0	0	0	0	0	0

[a] Fluid was subjected to heating at 85 °C at pH 1 for 10 min and subsequently readjusted to pH 7.5 for analysis.
[b] Eggs were allantoically inoculated with approximately 25 $TCID_{50}$ of Turkey virus or 40 $TCID_{50}$ of the other viruses.
[c] Turkey virus allantoic fluid was harvested at 24 hr p.i.
[d] Fluid was preincubated with the protease for 1 hr at 35 °C prior to the addition of substrate.

1 summarizes the results of this experiment. All nontreated viral fluids were unable to reduce markedly the protease activity. But heating at pH 1 followed by fluid adjustment to pH 7.5 enhanced the inhibitory capability especially of the 48 hr fluid samples. With 48 hr fluids of PR, FM and Brazil strains, there was a 2-fold rise in the degree of inhibition when compared to 24 hr fluids. In contrast, 24 hr Bangkok and Sendai infectious fluids were as effective as 48 hr fluids in suppressing the activity of the initial protease peak. These findings showed that differences did exist in the level of inhibition after acid-heat treatment of various 24 and 48 hr influenza and parainfluenza virus-infected allantoic fluids.

The ability of heated-acidic 48 hr influenza virus infected fluids to block the activity of several different proteases was examined. The percent inhibition of trypsin, chymotrypsin, subtilisin, *Streptomyces griseus* protease and 48 hr control egg allantoic and amniotic fluid proteases is compared in Table 2. Based on relative fluorescence values, the concentration of mammalian and bacterial proteases used was adjusted to correspond to the level observed in the initial protease peak. Egg embryonal fluids were used as harvested. Neither trypsin, chymotrypsin nor amniotic fluid protease levels were affected by preincubation with any of the viral fluids. Similarly, with the exception of PR virus fluid sample, no inhibitory effect was observed by viral fluids against *Streptomyces griseus* protease activity. Substantial inhibition of both 48 hr control allantoic fluid and subtilisin proteases did occur when acid-heated fluids of the various strains were tested. The protease inhibitory ability of these treated fluids appears to be specific for certain proteolytic enzymes.

Discussion

The data presented demonstrate the diverse effect various influenza A strains have on host neutral protease activity. Although this study was based on a relatively small number of virus strains, we found that the allantoic fluid proteolytic activity produced in eggs by the strains tested fell into one of three basic patterns. However, in all profiles the observed periodic peaks of enzyme was always followed by a partial shut down of protamine cleaving proteases. Comparison of the characteristic profiles generated and the time of partial shut down of proteases by different virus strains shows that similarities exist between PR and FM, Brazil and Ontario/Turkey and Bangkok and Sendai strains. These studies imply that influenza virus subtype relationships cannot be linked with the basic pattern of proteolytic activity observed. Whether other influenza strains are capable of inducing allantoic fluid protease profiles that differ from those obtained remains to be determined.

The evidence that inhibitors in highly infectious fluids were involved in reducing the activity of protamine cleaving proteases was provided by mixing acid-heat treated 48 hr PR virus allantoic fluid with allantoic fluid obtained from the initial protease peak. This enzyme activity was inhibited substantially by the 48 hr virus fluid. Similar analysis of 48 hr virus-infected allantoic fluids of different influenza strains showed that protease activity can be inhibited although to varying degrees. Differences found in the inhibitory capacity of virus fluids may imply that optimal conditions for specific strains were not used. Since protease levels of 24 hr virus-infected fluids of H1N1 influenza strains were well below control levels, it was unexpected that acid-heat treatment failed to increase their inhibitory capacity to the levels observed with treated 48 hour virus fluids. There are several possible explanations to account for this finding. (i) Possibly less inhibitor was present at 24 hr. (ii) The assay procedure for measuring inhibition was not sensitive enough to adequately detect the level of inhibition. (iii) The inhibitor(s) present at 24 hr was more susceptible to inactivation by the acid-heat treatment used for 48 hr virus fluids. Because of the findings with 24 hr virus fluids, allantoic fluids harvested at earlier intervals were not analyzed for the presence of inhibitor(s). Consequently, it was not possible to determine whether protease inhibitors are released intermittently into the allantoic fluid during the course of virus replication. It is possible, however, that the periodic oscillations observed early in virus infection may be a result of periodic depletion of allantoic fluid inhibitor pools.

The specificity of influenza virus induced allantoic fluid inhibitor(s) described here differs from the trypsin inhibitor in 17-day-old amniotic fluid (Bainter and Feher, 1973) and ovomucoid in chicken egg white (Lineweaver Murray, 1947). Allantoic fluid protease inhibitor had properties similar to those of subtilisin inhibitors reported for oyoinhibitor (Matsushima, 1958) and ovomacroglobulin (Kitamoto *et al.*, 1982) from chicken eggs, black bean seeds (Seidl *et al.*, 1982) and culture filtrates of *Streptomyces albogriseolus* (Ikenaka *et al.*, 1974). Isolation of subtilisin inhibitor(s) from influenza

virus-infected allantoic fluids should be useful for comparison with those isolated from avian, plant and bacterial sources.

Although the role of thermostable protease inhibitors in the influenza virus replicative cycle is unknown, studies on adenovirus (Levine and Ginsberg, 1967), vesicular stomatitis virus (Baxt and Bablanian, 1976) and herpes simplex type 2 (Hill *et al.*, 1983) indicate that they rely on virus specified inhibitors to block critical events in the metabolism of infected cells. The results described in this report prompts us to speculate that virus induced inhibitors present in highly infectious allantoic fluid block host protease activity to protect influenza virus proteins from any further proteolytic hydrolysis. Supporting this speculation, polyacrylamide gel electrophoretic studies have shown influenza virus polypeptides to be susceptible to degradation by proteases with different specificities (Sabina *et al.*, 1981). If allantoic fluid proteases play an essential role early in the course of influenza virus infection, then as the infectivity titer rises, it seems probable that thermostable inhibitors are involved in blocking protease activity to prevent continued degradation of virus polypeptides. Whether changes in the synthesis or degradation of such inhibitors are important during the influenza virus replicative cycle remains to be explored.

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