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EFFECTS OF INFLUENZA VIRUS REPLICATION ON NEUTRAL PROTEOLYTIC ACTIVITIES IN EMBRYONATED EGG FLUIDS

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Summary. — The levels of neutral protease activity associated with allantoic and amniotic fluids of embryonated eggs during the replication of influenza strains A/PR/8/34 (H1N1) and A/turkey/Ontario/7732/66 (H5N9) were investigated. A sensitive fluorometric technique proved useful for characterization and monitoring changes of protease activities in egg fluids. The predominant type of protease in allantoic and amniotic fluids had trypsin-like specificities. Variation in protease levels of both fluids occurred throughout the course of virus replication irrespective of the virus strain or the route of inoculation used. Concomitant with the production of high levels of infectious virus there was a marked decrease in neutral protease activity in the fluid from the cavity initially infected. Translocation of virus also occurred especially with amniotically infected eggs, as evidenced by high infectious virus titers and decreased protease activities in allantoic fluids.

Key words: *influenza A viruses*; *neutral proteases*; *embryonated egg fluids*

Introduction

Influenza virus infectivity is dependent upon the hemagglutinin (HA) glycoprotein being cleaved by proteases into the subunits HA_1 and HA_2 (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975). It has been suggested that viral strain-specificity and host-cell type account for differences in the proteolytic hydrolysis of HA (Lazarowitz *et al.*, 1973; Bosch *et al.*, 1981). An enzymic cleavage mechanism for modifying influenza HA has been proposed by Bosch *et al.*, (1981) and Garten *et al.*, (1982). Their data indicate that initially the cleavage site of the HA reacts with a trypsin-like endoprotease supplied by host cells. An exopeptidase, presumably virus-specified subsequently eliminates arginine from the cleavage site which results in fully infectious virus.

Since influenza A strains multiply with infectious virus formation in embryonated chicken eggs (Klenk *et al.*, 1975), it could be expected that this

host system possesses neutral proteases of trypsin-like specificity to cleave HA molecules. The lack of quantitative information on levels of neutral protease activity in embryonal fluids of influenza virus-infected and uninfected eggs prompted this study. Analysis of proteolysis can be achieved through the use of sensitive fluorometric techniques which are suitable for the assay of nanogram quantities of proteases (Brown *et al.*, 1973; Garese *et al.*, 1979). Therefore, we undertook to investigate the effect of replication of two strains of influenza A virus on neutral protease activities associated with allantoic and amniotic fluids of embryonated eggs.

Materials and Methods

Viruses. Influenza strains A/PR/8/34 (H1N1) (PR) and A/turkey/Ontario/7732/66 (H5N9) (TU) were provided by D. A. McLeod, Laboratory Centre for Disease Control, Ottawa, Canada and G. Lang, Ontario Veterinary College, Guelph, Canada, respectively. Both strains were grown for 48 hr at 37 °C in 9-day-old embryonated chicken eggs inoculated allantoically with diluted virus seed (10^{-3} for PR and 10^{-5} for TU). After clarification of harvested allantoic fluids, virus was pelleted by ultracentrifugation at 65,000 × g for 45 min followed by resuspension in tryptose phosphate broth and then stored at −60 °C.

Cells. Chick embryo (CE) cells were prepared by collagenase-trypsin treatment of 10-day-old embryos. For virus assays, enzyme-dispersed cells were resuspended in Medium 199 supplemented with 3% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml) (M199-3FCS).

Virus assays. A microtiter system adapted from Moore (1977) was used for the 50% endpoint assay of infectious virus. Serial tenfold dilutions of virus were made in M199-3FCS. In wells of Microtest II Falcon plates, 25 μl samples of viral dilutions were incubated with 125 μl of cell suspension containing 4×10^4 CE cells. After 4 days incubation at 37 °C in an atmosphere of 5% CO_2 in air, the tissue culture 50% infectious dose ($TCID_{50}$) was estimated according to Spearman-Kärber (Finney, 1964). The hemagglutinating activity of viruses was determined with rooster red blood cells by the method of Palmer *et al.*, (1975).

Neutral protease assay. The assay method was a modification of that described by Brown *et al.*, (1973). Prior to enzyme assay, test embryonal fluids were clarified by centrifugation at 2,200 × g for 10 min. One hundred μl of fluid was incubated with an equal volume of substrate solution (1% in 0.1 mol/l sodium phosphate buffer, pH 7.5) for 5 hr at 35 °C. A 50 μl aliquot was taken and added to 3 ml of 0.1 mol/l sodium phosphate buffer, pH 7, followed by the addition of 500 μl of 0.02% fluorescamine in acetone. Fluorescence was measured in a Turner Model 430 spectrofluorometer with the wavelengths of excitation and emission at 390 nm and 475 nm, respectively. Values obtained were corrected by subtraction of the fluorescence contributed by the substrate and fluid (enzyme) blanks. Fluid protein was assayed by the Bradford procedure (1976) with bovine serum albumin as standard.

Chemicals. Casein (hammersten grade) was purchased from BDH Chemicals Ltd., Poole, England and beta casein from OCN Pharmaceutical Inc., Cleveland, Ohio. Crystalline trypsin (EC.3.4.21.4) and all other chemicals were obtained from Sigma Chemical Co., St.Louis, Mo.

Results

Before monitoring the changes of neutral proteolytic activity in egg fluids during the replication of influenza virus, it was important to establish the optimal conditions for the fluorometric assay of enzymes. Protamine sulfate from herring, a known ideal substrate for trypsin-like enzymes (Garesse *et al.*, 1979) was chosen for optimization experiments. Proteolytic activities in both allantoic and amniotic fluids from 9-day-old embryonated eggs were

Table 1. Ability of allantoic and amniotic neutral proteases to cleave different substrates

Substrate	Protease activity (relative fluorescence/μg protein)	
	Allantoic fluid	Amniotic fluid
Protamine sulfate (from herring)	1.0	11.5
Protamine sulfate (from salmon)	0.54	7.5
Bovine albumin	0.29	1.0
Casein (hammersten grade)	0.33	0.25
Beta casein	0.17	0.5

liner with time, reaching maximum levels after 5 hr of incubation at pH 7.5 (data not shown). Similarly, there was a linear relationship between the amount of fluid protein used and the increase in relative fluorescence (Fig. 1-I and II). As shown in the insert of Fig. 1-I, the standard curve for trypsin exhibits the linearity requirements of a valid assay under the conditions adopted.

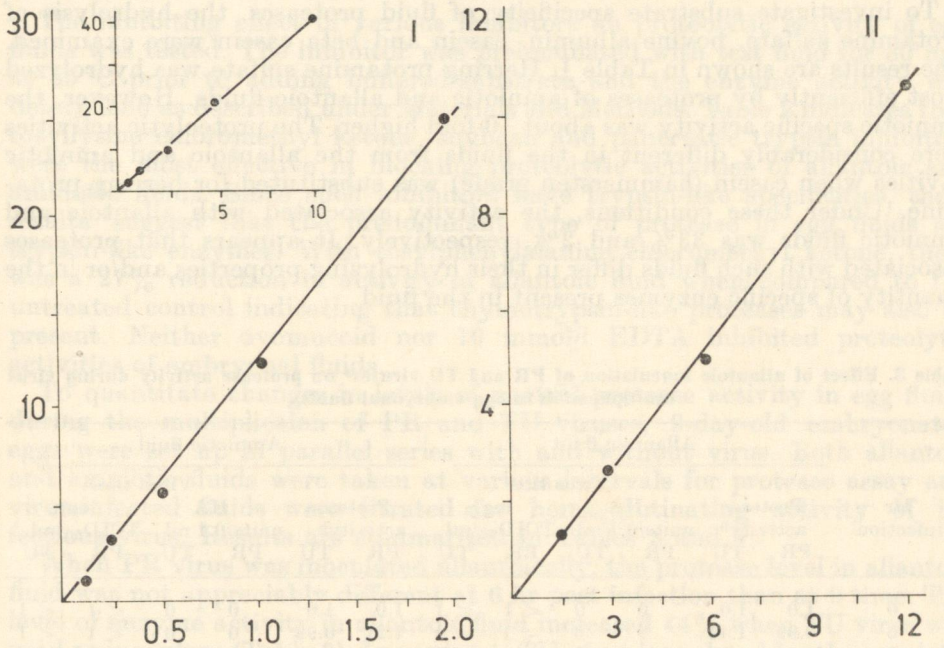

Fig. 1.

Standard curves for assay of neutral protease activity in amniotic (I) and allantoic (II) fluids Each test sample was incubated with a buffered preparation of protamine sulfate (herring) at pH 7.5 and 35 °C. Fluorescamine was added to quantify the release of free amino groups from protamine. The insert in Fig. 1-I shows the assay of trypsin under conditions described above: abscissa; μg trypsin ($\times 10^{-2}$)
Abscissae; μg protein; ordinates: relative fluorescence

Table 2. Influence of various inhibitors on neutral protease activity in embryonal fluids

Inhibitor	Final conc.	Activity (%)* Allantoic fluid	Amniotic fluid
None	—	100	100
Phenylmethylsulfonyl fluoride	2 mmol/l	50	46
Tosylphenylalanine chloromethyl ketone	4 mmol/l	73	93
Tosyllysine chloromethyl ketone	4 mmol/l	27	7
EDTA	10 mmol/l	100	100
	100 mmol/l	72	73
Ovomucoid (ovoinhibitor free)	2 mg/ml	100	100
Ovomucoid	2 mg/ml	100	100
Pancreatic trypsin inhibitor	2 mg/ml	17	15
Soybean trypsin inhibitor	1 mg/ml	3	21

* All inhibitors were preincubated with the embryonal fluids for 30 min at 35 °C before adding the substrate.

To investigate substrate specificity of fluid proteases, the hydrolysis of protamine sulfate, bovine albumin, casein and beta casein were examined. The results are shown in Table 1. Herring protamine sulfate was hydrolyzed most efficiently by proteases of amniotic and allantoic fluids. However, the amniotic specific activity was about 10-fold higher. The proteolytic activities were considerably different in the fluids from the allantoic and amniotic cavities when casein (hammersten grade) was substituted for herring protamine. Under these conditions, the activity associated with allantoic and amniotic fluids was 33% and 2%, respectively. It appears that proteases associated with such fluids differ in their hydrolyzing properties and/or in the quantity of specific enzymes present in the fluid.

Table 3. Effect of allantoic inoculation of PR and TU viruses[a] on protease activity during virus multiplication in egg embryonal fluids

Hr infection	Allantoic fluid: Protease activity[b] PR	TU	Virus titer: HA units/0.1 ml PR	TU	$\log_{10}$ $TCID_{50}$/ml PR	TU	Amniotic fluid: Protease activity[b] PR	TU	Virus titer: HA units/0.1 ml PR	TU	$\log_{10}$ $TCID_{50}$/ml PR	TU
0	1.0	1.0	0	0	< 1	< 1	1.0	1.0	0	0	< 1	< 1
6	0.99	1.44	0	0	< 1	< 1	1.21	0.98	0	0	< 1	< 1
12	1.0	0.88	160	0	5.35	3.8	1.2	1.11	0	0	1	3.3
24	0.41	0.55	640	'160	6.35	7.6	0.93	1.07	0	0	3.1	4.3
48	0.41	0.34	1280	320	6.6	7.6	1.06	ND[c]	160	10	4.1	4.8

[a] Inoculum of PR and TU viruses used was ~ 6 $TCID_{50}$/egg.

[b] Ratio of enzyme activities of virus-infected fluid to uninfected fluid based on relative fluorescence/μg fluid protein.

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

Table 4. Effect of amniotic inoculation of PR and TU viruses[a] on protease activity during virus multiplication in egg embryonal fluids

Hr post infection	Amniotic fluid						Allantoic fluid					
	Protease activity[b]		Virus titer HA units/0.1 ml		Virus titer $\log_{10}$ $TCID_{50}$/ml		Protease activity[b]		Virus titer HA units/0.1 ml		Virus titer $\log_{10}$ $TCID_{50}$/ml	
	PR	TU	PR	TU	PR	TU	PR	TU	PR	TU	PR	TU
0	1.0	1.0	0	0	< 1	< 1	1.0	1.0	0	0	< 1	< 1
6	1.06	0.91	0	0	< 1	< 1	1.26	0.92	0	0	< 1	< 1
12	0.29	1.04	160	0	6.6	5.6	0.95	1.12	4	0	3.6	3.6
24	0.28	0.42	1280	160	6.8	7.6	0.58	0.74	640	80	4.6	6.3
48	ND[c]	ND[c]	2560	320	6.6	7.6	0.36	0.29	1280	160	5.1	7.3

[a] Inoculum of PR and TU viruses used was ~ 4 $TCID_{50}$/egg.

[b] Ratio of enzyme activities of virus-infected fluid to uninfected fluid based on relative fluorescence/μg fluid protein.

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

The inhibiting effect of various inhibitors on proteolytic activity of egg fluids was tested. The inhibitor was preincubated with test fluid for 30 min at 35 °C prior to adding buffered-substrate and the enzyme activity was determined as described under Materials and Methods. Table 2 indicates that tosyllysine chloromethyl ketone, soybean and pancreatic trypsin inhibitors were the most effective in blocking proteolytic activities of allantoic and amniotic fluids. Since such inhibitors have trypsin-like specificities, these results suggest that the predominant type of protease in egg fluids are trypsin-like enzymes. With tosylphenylalanine chloromethyl ketone, there was a 27% reduction in activity of allantoic fluid when compared to the untreated control indicating that chymotrypsin-like proteases may also be present. Neither ovomucoid nor 10 mmol/l EDTA inhibited proteolytic activities of embryonal fluids.

To quantitate changes in levels of neutral protease activity in egg fluids during the multiplicaion of PR and TU viruses, 9-day-old embryonated eggs were set up in parallel series with and without virus. Both allantoic and amniotic fluids were taken at various intervals for protease assay and virus-infected fluids were titrated for hemagglutinating activity of infectious virus. Results are summarized in Tables 3 and 4.

When PR virus was inoculated allantoically, the protease level in allantoic fluid was not appreciably different at 6 hr post infection than at 0 time. The level of enzyme activity in allantoic fluid increased 44% when TU virus was used as inoculum (Table 3). In contrast, TU virus inoculated by the amniotic route did not enhance the protease activity in amniotic fluid after 6 hr of infection (Table 4). Such findings suggest that viral strain differences can influence proteolytic activities in egg fluids. By 24 hr, a considerable reduction in protease levels of allantoic and amniotic fluids was obtained irrespective of the virus strain used. The fact that almost maximum infectious virus titers were reached within 24 hr could account for the drop-off in enzyme activity.

Since TU virus 7732 is a velogenic strain (Lang *et al.*, 1968), it was desirable to determine whether the viral route of inoculation had any effect on the proteolytic activity of fluid in an adjacent egg cavity. Allantoic inoculation of TU and PR viruses did not significantly alter the protease level in fluids from the amniotic cavity throughout the test period. However, relatively low hemagglutinating activity and infectious virus titers were demonstrable (Table 3). If viruses were inoculated by the amniotic route, a substantial decrease in the enzyme level of allantoic fluids was noted by 48 hr post infection (Table 4). Also, comparable viral titers in both fluids occurred only when the TU strain was used. Apparently the magnitude of virus translocation from one to another fluid cavity during the course of infection is much greater in eggs infected amniotically than by the allantoic route.

Discussion

The detection and partial characterization of neutral proteases in the allantoic and amniotic fluids of embryonated eggs was possible by use of a sensitive fluorometric assay. Proteolytic enzymes with trypsin-like specificity were predominant in both embryonal fluids although the specific activity of amniotic fluid was substantially higher. Interestingly, allantoic and amniotic fluid proteases cleaved herring protamine most efficiently while limited hydrolysis of salmon protamine was observed. Differences in primary structure between the two protamines may account for this variability in the extent of proteolytic degradation (Busch, 1965).

Comparative enzyme studies during the first 12 hr of multiplication of PR and TU viruses demonstrate variability in the levels of fluid protease activity. However,by 24 hr post infection there was a consistently marked decrease in proteolytic activity of fluids harvested from cavities initially infected irrespective of the route of inoculation or virus strain used. This was paralleled by the production of near maximal yields of HA and infectious virus. A factor possibly responsible for decreased protease activity is the amount of infectious virus present in fluids.

According to Lang *et al.* (1968), the velogenic TU virus diffuses rapidly throughout the embryo. Although infectious virus of both strains was detected in the amniotic fluid after allantoic inoculation, such low virus titers were apparently not sufficient to depress proteolytic activity. However, the degree of viral translocation following amniotic infection resulted in sufficient yields of infectious virus necessary to markedly reduce allantoic protease activity. These experiments strongly suggest that virus can be more readily translocated when the embryo is inoculated by the amniotic route. Although it cannot be ruled out that the procedure used for inoculating virus may have led to contamination of the allantoic fluid.

The reason for the observed decrease in protease levels is currently unknown. As proposed by Garten (1982) two proteases are believed to be involved in the cleavage mechanism of the influenza virus HA. Prior to the production of high titers of infectious virus, the major type of protease may be the host specified trypsin-like endoprotease which can efficiently hydrolyze

herring protamine. By 24 hr post infection a virus specified protease with reduced ability to cleave herring protamine may predominate. Further analysis of infectious fluids for the presence of virus specified proteases and for either existing or newly synthesized protease inhibitors that may be involved in the observed partial shut down of enzyme activity are in progress.

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