CHANGE OF ENZYME ACTIVITIES DURING THE EARLY STAGE OF INFLUENZA VIRUS INFECTION

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SUMMARY: Intracellular activities of various hydrolytic enzymes were investigated in monkey kidney cells infected with the A/NWS strain of influenza virus. At the early stage of infection, there was a significant decrease in the activity of β-D-galactosidase, α-D-mannosidase, N-acetyl-β-D-glucosaminidase, acid and alkaline phosphatase. The decrease was roughly proportional to the multiplicity of infection, and restored at 2 hr after the infection. Corresponding to this intracellular decrease, there was an increase in the activities of these enzyme outside the cells. The results suggested that these hydrolytic enzymes would be released from the cell membrane or the lysosomes near the membrane in the process of adsorption and penetration of the virus particles.

There is still little information concerning the biochemical change which occurs on or near the cell surface during the early stage of virus infection. In a previous paper (1) we demonstrated that adsorption of denatured virus particles to the cell surface causes a significant increase in the amount of sialic acid residues which are accessible to the action of sialidase of Cl. perfringens. This increase suggests the occurrence of some rearrangement among membrane components. It can be thought that this kind of rearrangement might alter the location or the total activities of the enzymes which exist in the cell membrane or which are sequestered in lysosomes in the cell periphery. In the present communication, the intra- and extracellular activities of various hydrolytic enzymes were investigated in the early stage of influenza virus infection to monkey kidney (MK) cells.

MATERIALS AND METHODS

Virus strain and infection: The A/NWS (HONI) strain of influenza virus was propagated in 10-day-old embryonated eggs. The virus in the chorioallantoic fluids was concentrated by differential centrifugation and purified by from 10 to 50% linear sucrose gradient centrifugation (2,3). Confluent MK cultured were infected with different multiplicity of infection (m.o.i.) of the purified virus in 10 ml Medium 199 and placed at 37°C. At various intervals, each group of cultures were washed twice with phosphate buffer saline (pH 7.2). Thereafter

the cells were harvested with a rubber policeman and sedimented by centrifugation at 2,000 rpm for 10 min.

Cell culture: Primary MK cells (3.5×10^5 cells per ml) suspended in 50 ml of LE growth medium containing 5% calf serum were placed in each rubber-stoppered bottles (10 x 16 cm) and incubated at 37°C. After 3 days, the growth medium was replaced by Medium 199 containing 5% calf serum. L cells were grown as described previously (1).

Preparation of materials for determination of the intra- and extracellular enzyme activities: After incubation of cells, the medium was with drawn and lyophilized. A suitable volume of distilled water was added before the determination of the enzyme activities. The intracellular activity was determined as described by Bosman (4). The cells were homogenized at 0°C with 30 vol. of 0.1% Triton X-100 by 20 strokes in Dounce homogenizer. The homogenate was kept for 16 hr at 4°C under stirring and then centrifuged at 30,000 x g for 1 hr. The supernatant was lyophilized and made to a suitable volume with distilled water. Total protein was determined by the procedure of Lowry (5).

Substrates: p-Nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-β-D-glucopyranoside and p-nitrophenyl-α-D-mannopyranoside were purchased from Calbiochem., U.S.A. 4-Nitrophenyl-β-D-galactopyranoside and 4-nitrophenyl-N-acetyl-β-D-glucosaminide were purchased from BDH chemicals Ltd., England. Phenolphthalein-glucuronic acid, p-nitrophenyl phosphate and bovine hemoglobin were purchased from Koch-Light Laboratories Ltd., England, Sigma Chemical Co., U.S.A., and Daiichi Pure Chemical Co., Ltd., Japan, respectively.

Determination of enzyme activities: The glycosidase activity was determined using p-nitrophenyl derivatives by the following procedure. A solution containing 0.05 ml of 0.05M p-nitrophenyl derivatives, 0.4 ml of 0.05M citrate phosphate buffer (pH 4.2) and 0.05 ml of a test material were incubated for 1 hr at 37°C. The reaction was terminated by the addition of 0.2 ml of 0.4M glycine-NaOH buffer (pH 10.5), and the mixture was centrifuged at 3,000 rpm for 5 min, and the hydrolysis product, p-nitrophenol in the supernatant, was measured at 400 nm. The activity of β-glucuronidase was determined as follows. A mixture consisting of 0.05 ml of 0.01M phenolphthalein-glucuronic acid, 0.4 ml of 0.1M sodium acetate (pH 5.0) and 0.05 ml of a test material was incubated for 1 hr at 37°C. The reaction was terminated by the addition of 1.5 ml of 0.5M glycine-NaOH buffer (pH 10.5), and the reaction mixture was centrifuged at 3,000 rpm for 5 min, and the phenolphthalein in the supernatant was measured at 550 nm. The activity of acid and alkaline phosphatase and cathepsin D was determined by methods described previously (6,7).

RESULTS

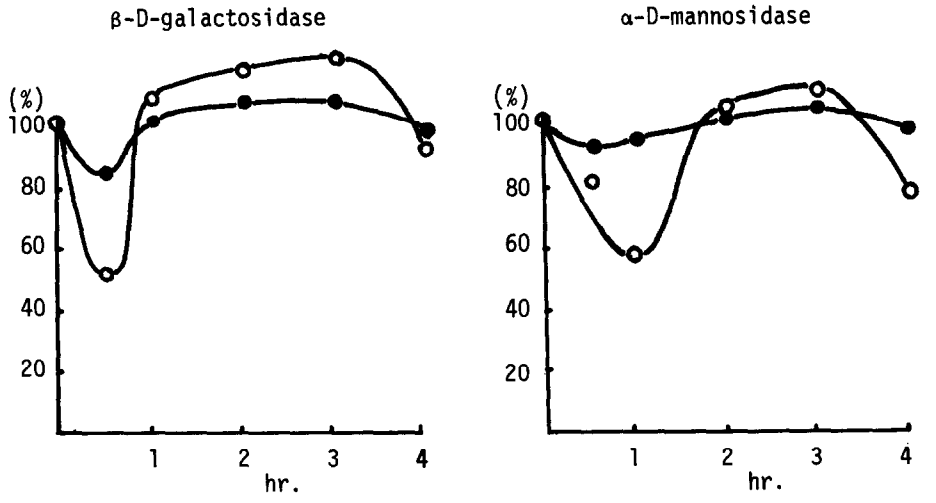
Change in enzymatic activities after virus infection: When MK cells were infected with the input multiplicity of 3 per cell of influenza virus A/NWS strain, there was a decrease in the intracellular activity of β-D-galactosidase and α-D-mannosidase at 30 to 60 min after infection (Table 1). In this condition, sialidase activity was detected inside the cells at 6 hr after infection, and the viral multiplication in the cells was confirmed as described previously (8). The result in Table 1 were plotted in a graph and are shown in Fig. 1 together with the enzyme activities in L cells after the infection. The activities of these two enzymes were restored to the original levels at 2 hr after the infection. A similar pattern was observed also in case of L cells, but the change was less clear.

Table 1. Change of the activity of β-D-galactosidase and α-D-mannosidase in MK cells after the infection with influenza virus (A/NWS)

Enzymes	The product of the enzyme reaction, p-nitrophenol n moles/100 μg protein (hr)					
	0	0.5	1	2	3	4
β-D-galactosidase	67.4	33.0	72.2	80.5	82.6	60.3
α-D-mannosidase	41.6	35.0	23.5	42.4	44.9	30.4

Effect of multiplicity of infection on the descrease of the intracellular enzymatic activities: In order to check whether the decrease in the activities of the two enzymes described above is due to the viral infection, the number of infective viral particles for infection was varied from 0.1 to 4.0 m.o.i.,

Fig. 1. Time course of the enzymatic activity in MK and L cells



The infected and control cells were harvested at the indicated time and the intracellular activities of the two enzymes were determined. The activity at each point was expressed as the percentage of that to the control cells.

o——o MK cell ●——● L cell

and the intracellular activities of these and the other hydrolytic enzymes were investigated in MK cells.

As shown in Table 2, there was a significant decrease in the activities of β-D-galactosidase, α-D-mannosidase, N-Ac-β-D-glucosaminidase, acid and alkali phosphatase at 30 min after the infection when m.o.i. was varied from 1.0 to 4.0. In all cases at 3 hr the enzyme activities were restored to the original level. Table 2 shows that there is a significant decrease in the intracellular activities of these enzymes when the cells were infected with virus particles of not less than 1.0 m.o.i.

In another experiment, m.o.i. was raised up to 100 and both the intra- and extracellular enzyme activities were determined at 40 min after infection. As shown in Table 3, the larger the m.o.i., the decrease in the intracellular enzyme activities was more marked. When the enzyme activities in the medium were determined and were added to the intracellular activities to make the total activities per bottle, there was not a significant change in the total activities. The result indicates that various hydrolytic enzymes are released from the host cells into the medium during the early stage of virus infection.

DISCUSSION

As reported in a previous paper (1), the binding of denatured influenza virus to MK cells increases the amount of sialic acid residues which are removed by the action of Cl. perfringens sialidase, suggesting the occurrence of a rearrangement of components which constructs the membrane or the cell surface. It will be argued that infective viral particles which bind to the cell surface and penetrate into the cytoplasm would cause more drastic changes in the membrane structure or the structures near the membrane. It is possible that such a change disturbs the location of various enzymes which exist on or near the cell membrane. The results described in this paper indicates that at about 30 or 40 min after the infection, the activities of β-D-galactosidase, α-D-mannosidase, N-acetyl-β-D-glucosaminidase, acid and alkaline phosphatase decrease significantly, and the grade of decrease is more marked with the

Table 2. The intracellular activity of various enzymes in MK cells after infection

After infection	30 min.				3 hr
Enzymes m.o.i.	0	0.1	1.0	4.0	4.0
α-D-Glucosidase	10.9 ± 1.4	10.6 ± 4.8	9.2 ± 1.4	8.1 ± 1.5	11.2 ± 1.7
β-D-Glucosidase	8.7 ± 1.0	8.2 ± 1.0	8.1 ± 0.9	6.3 ± 0.9	9.1 ± 1.1
β-D-Galactosidase	57.1 ± 4.9	55.3 ± 5.1	44.9 ± 3.4	37.5 ± 3.5	60.2 ± 4.6
α-D-Mannosidase	43.8 ± 2.5	41.6 ± 2.4	40.5 ± 1.8	33.4 ± 1.3	41.3 ± 3.8
N-Acetyl-β-D-glucosaminidase	569 ± 48	558 ± 45	530 ± 14	485 ± 47	541 ± 47
Acid phosphatase	400 ± 27	406 ± 26	398 ± 25	367 ± 15	383 ± 21
Alkali phosphatase	71.8 ± 3.8	70.5 ± 3.8	66.8 ± 4.6	53.2 ± 4.8	65.8 ± 4.2
β-Glucuronidase	8.3 ± 0.7	8.3 ± 0.7	7.8 ± 0.6	6.9 ± 1.3	7.6 ± 1.1
Cathepsin D	0.182 ± 0.01	0.180 ± 0.01	0.167 ± 0.01	0.16 ± 0.01	0.185 ± 0.01

Results are expressed as n moles hydrolyzed substrate/hr/100 μg protein. OD at 280 nm is used in the case of cathepsin D. Each figure was obtained from the average of six separate determinations together with S.D.

m.o.i.: multiplicity of infection of plaque forming unit.

Table 3. Effect of m.o.i. on the change of intracellular and total activity of various enzymes

Enzymes \ m.o.i. / Fractions	0		1.0		10		50		100	
	Cell	Total	Cell	Total	Cell	Total	Cell	Total	Cell	Total
α-D-Glucosidase	390	390	316	316	257	341	225	440	213	484
β-D-Glucosidase	242	243	219	219	159	159	159	159	150	150
β-D-Galactosidase	1601	1674	1325	1476	1159	1425	1044	1369	918	1332
α-D-Mannosidase	974	1042	767	990	706	903	762	997	778	1002
N-Acetyl-β-D-glucosaminidase	19125	20360	15940	17970	13980	16440	13840	16550	14130	16780
Acid phosphatase	9716	9891	9367	9671	8612	9147	8399	9132	7908	9095
Alkali phosphatase	1849	1849	1477	1590	1005	1147	1034	1235	1020	1348
β-Glucuronidase	95.2	95.2	91.8	91.8	83.6	83.6	77.4	77.4	76.8	76.8
Cathepsin D	4.47	4.47	4.23	4.23	4.16	4.16	4.17	5.14	4.13	5.09

Results expressed as n moles/hr/bottle.

Viral infection was allowed to proceed for 40 min at 37°C. Each figure represents the average of triplicated experiments.

increase of the number of infective viral particles. Our results also suggested that this decrease would be due to release of such enzymes to the medium, because the sum of the intra- and extracellular activity of each enzyme was almost constant. While our results clearly indicates that the enzyme release is really induced by the adsorption of virus particles or in the early stage of virus infection, it is also reasonable to assume that the released enzymes themselves might react with the components on the cellular membrane and thus play an active role in the penetration of virus particles.

In our experiments the decrease in the intracellular enzymes was restored to the original level at 2 hr after the infection. The recovery may be in relation to the further process of virus multiplication, especially to the uncoating process. Thus, the analysis of these hydrolytic enzymes are thought to give informations useful in clarifying the mechanism of virus adsorption and penetration and furthermore the membrane events during the virus infection.

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