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HEMOLYSIS AND FUSION BY INFLUENZA VIRUSES WITH HEAT-INACTIVATED NEURAMINIDASE ACTIVITY

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SUMMARY Influenza virus hemolytic activity was found to be more heat-resistant than neuraminidase activity, and to be heat-inactivated similarly to hemagglutination activity, in sharp contrast to the case with paramyxoviruses, where the hemolytic activity is the most heat-labile, and hemagglutination and neuraminidase activities are inactivated similarly by heat. Influenza viruses with heat-inactivated neuraminidase activity, which still showed hemagglutination and hemolytic activities, were found to be able to induce cell fusion and envelope fusion. This finding suggested that the hemolytic and fusion activities are not dependent on neuraminidase activity. The hemolytic activity was largely inhibited by anti-hemagglutinin serum of the same subtype. The pH range, heat stability, and antiserum susceptibility of the hemolytic activity were found to be independent of the cells in which the virus was grown.

INTRODUCTION

Various envelope viruses, including paramyxo- (Hosaka, 1958; Choppin and Compans, 1975; Ishida and Homma, 1978), orthomyxo- (Maeda and Ohnishi, 1980; Huang et al., 1981; Lenard and Miller, 1981), toga- (Karabatsos, 1963, 1965; Väänänen and Kääriäinen, 1979, 1980), and rhabdo- (Ohuchi et al., 1981) viruses, have been reported to have hemolytic activity, although the optimal pH for the hemolysis varies depending on the virus. The hemolyse by paramyxo- (Howe and Morgan, 1969; Apostolov and Almeida, 1972; Homma et al., 1976; Shimizu et al., 1976; Hosaka and Shimizu, 1977; Ishida and Homma, 1978) and by toga- (Helenius et al., 1980; White et al., 1980; Väänänen et al., 1981; Lenard and Miller, 1981) viruses are thought to be mediated by envelope fusion. Recently, morphological evi-

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dence that influenza virus hemolysis is mediated by envelope fusion was presented (Maeda et al., 1981; Yasuda et al., 1982). Thus, all the envelope viruses so far tested were found to have hemolytic activity and to cause envelope fusion in favorable conditions. The mechanism of viral hemolysis seems to be mostly common with that involved in envelope fusion, the step of penetration of virus.

Hemolysis by influenza virus was manifested with cleavage (HA_1+HA_2) of the hemagglutinin (HA_0) of tissue culture-grown influenza virus by trypsin treatment, suggesting that the hemolytic activity was associated with the hemagglutinin protein (Maeda et al., 1981). Huang et al. (1980a, b) reported that influenza virosomes fused with cell membranes at neutral pH, and neuraminidase played a role in the fusion.

We found that when influenza virus neuraminidase activity was heat-inactivated, the virus still retained hemolytic activity. The present report describes the heat stability of influenza virus hemolytic activity, its inhibition by monospecific antisera, and fusion and some other biological properties of virus with heat-inactivated neuraminidase activity. Studies on the influence of the host cells in which the virus was grown on the hemolytic activity are also reported.

TABLE 1. *Viruses used in the present study*

Virus	Abbreviation	HA, NA subtype	Egg passage number	Donor
[Influenza viruses]				
A/PR/8/34	PR8	H0N1	>100	(this laboratory)
A/WSN/33	WSN	H0N1	10 after CEF[a] culture	Dr. A. Sugiura (Institute for Public Health, Tokyo)
A/FM/1/47	FM1	H1N1	N. K.[b]	Dr. W. Gerhard (Wistar Institute, Philadelphia)
A/Adachi/2/57	Adachi	H2H2	about 20	Dr. K. Nakamura (formerly in this Institute)
A/Aichi/2/68	Aichi	H3N2	about 20	Dr. K. Nerome
A/Kumamoto/1/76	Kum 2	H2N2	32	Messrs. T. Koide & F. Sadahiro (Kanonji Inst., RIMD Foundation)
A/Tokyo/6/73	Tokyo	H3N2	about 10	K. & S.
A/Kumamoto/22/76	Kum 3	H3N2	about 20	K. & S.
A/Bangkok/1/79	BKK	H3N2	15	Dr. K. Nerome
A/X-7 (recombinant, Kilbourne *et al.*, 1968)	X-7	H0N2	N. K.	Dr. Sugiura
B/Gifu/2/73	B/Gifu		about 10	K. & S.
B/Singapore/222/79	B/Sin		11	K. & S.
[Paramyxoviruses]				
Sendai virus, Z strain	SV		>300	(this laboratory)
Newcastle disease virus, Miyadera strain	NDV		N. K.	Dr. T. Kimoto (Osaka prefectural Institute of Public Health, Osaka)
Mumps virus, Urabe strain	MV		30 (AS[c])	Dr. K. Yamanishi (this Institute)

[a] Chick embryo fibroblasts.
[b] Not known.
[c] Amniotic sac.

MATERIALS AND METHODS

1. *Virus*

The viruses used are described in Table 1. They were all grown in the chorioallantoic cavity of 10- or 11-day-old chick embryos at 35 C for 3 days except mumps virus, which was grown in amniotic sacs of 8- or 9-day-old chick embryos at 34 C for 4 days. Harvested chorioallantoic fluids were clarified by centrifugation at $2,000 \times g$ for 15 min, and the supernatants were centrifuged at $40,000 \times g$ for 30 min. The pelleted virions were suspended in isotonic saline after careful removal of all the supernatant. These partially purified virions were again clarified by low speed centrifugation before use.

The WSN strain grown in MDCK cells, which were a gift from Mr. H. Okuno (this Institute), was also prepared. The virus was partially purified by differential centrifugation, and activated by trypsin (Choppin et al., 1975).

2. *Antisera*

All the anti-influenza virus HA and NA goat antisera used were kindly supplied from the Research Resources Branch of NIAID (NIH, Bethesda). The hemagglutination inhibition (HI) titers of these sera are shown in Table 2. The HI titers in the table were essentially consistent with data on combination of virus and anti-sera given in the Catalog of NIAID (1978–1980).

3. *Hemagglutination (HA) and hemagglutination inhibition (HI) assay*

These assays were done as described by Fazekas and Webster (1966), but on a microscale. Before the HI test, the sera were treated with trypsin-KIO_4 to remove non-specific inhibitors (Dowdle et al., 1979).

4. *Neuraminidase (NA) and neuraminidase inhibition (NI) assay*

NA activity was measured by the method of Aminoff (1961), but on half the scale of his original method. NI titers were measured by the method of Webster and Periera (1968). The NI titers of anti-neuraminidase sera are shown in Table 3.

5. *Hemolysis (HL) assay*

A sample of 25 μl of influenza virus in isotonic saline was mixed with 1 ml of 2% human erythrocyte (O-type) suspension in 0.85% NaCl solution containing 1/20 *M* sodium acetate buffer (pH 5.3) in the cold for 15 min, and incubated at 37 C for 60 min. Then 1 ml of phosphate buffer saline

TABLE 2. *HI titers of the antisera to influenza virus hemagglutinin and neuraminidase antigens*

Antiserum	Antigen viruses								
	PR8	WSN	X-7	FM1	Kum 2	Aichi	Tokyo	Kum 2	BKK
anti-HO (PR8)[a]	12800[b]	640	1280	160	80	40	40	40	<20
anti-H1 (FM1)	1280	160	160	12800	20	80	40	<20	<20
anti-H2 (Sing.)	160	160	80	80	1280	160	160	40	<20
anti-H3 (Aichi)	160	160	80	160	80	6400	1280	320	80
anti-N1 (Heq N1)	<20	<20	<20	<20	<20	<20	<20	<20	<20
anti-N2 (Sing.)	<20	<20	<20	80	40	<20	40	<20	<20
anti-HA/B (Lee)	<20	<20	<20	40	20	20	40	<20	20
anti-NA/B (Lee)	40	40	40	80	40	<20	80	<20	<20

[a] The parent strain from which the immunogen was derived.

[b] Solid and broken underlines indicate the homotypic and heterotypic inhibitions of HL, respectively, with in the same HA subtype, in terms of hemagglutinin.

TABLE 3. *NI titers[a] of anti-N1 and anti-N2 sera to various strains of influenza virus*

Antiserum	Antigen viruses								
	PR8	WSN	X-7	FM1	Kum 2	Aichi	Tokyo	Kum 3	BKK
anti-N1[b] (HeqN1)	68	73	73	64	60	60	60	60	60
anti-N2 (Sing.)	<30	<30	>200	<30	>200	46	<30	<30	<30
anti-NA/B (Lee)	<30	<30	<30	<30	<30	<30	<30	<30	<30

[a] See "Materials and Methods"

[b] Anti-N1 serum had an NI titer of 300 against homologous virus (A/NJ/8/76, (HON1)) NA activity (NIAID Catalog, 1978–1980), but NI titers of 60–70 against other A type influenza viruses, inclusive of viruses of the same NA subtype.

(PBS) (pH 7.2) was added to stop the reaction, and the hemoglobin content in the supernatant was determined spectrophotometrically at 540 nm. Percentage hemolysis was calculated on the basis of total lysis of erythrocytes with 1% Triton X-100. Hemolysis by paramyxoviruses was measured similarly, except that PBS (pH 7.2) was used instead of acetate buffered saline.

For determination of the pH range for hemolysis, human erythrocytes were suspended in saline containing 1/20 *M* acetate buffer of different pH values, allowed to react with virus, and measured as described above.

6. *Hemolysis inhibition (HLI) test*

A sample of 25 μl of influenza virus (2500–4000 HA) causing hemolysis giving an optical density of about 1.0 at 540 nm was mixed with equal volumes of 2-fold serial dilutions of antiserum at room temperature for 30 min, and then 1 ml of human erythrocyte suspension (pH 5.3) was added. Hemolysis was measured as described above. The reciprocal of the final dilution causing 50% inhibition of hemolysis was taken as the hemolysis inhibition titers (HLI).

7. *Cell fusion assay*

Chicken erythrocytes were used for cell fusion for convenience in identification of polykaryocytes containing a number of nuclei. A mixture of 50 μl of influenza virus, PR8 strain (16,000 HA/ml) and 100 μl of 2% chicken erythrocyte suspension in the same medium (pH 5.3) was incubated at 37 C for 60 min. Then the mixtures were examined for polykaryocytes under a light microscope. Fusion was determined only qualitatively, because many aggregated cells were formed during the incubation, and these prevented accurate measurement of the cell number.

8. *Immunoelectron microscopy*

The interaction of human erythrocytes and the WSN strain in which neuraminidase activity and infectivity had been inactivated by heating at 55 C for 30 min was studied by indirect immunoelectron microscopy. Under conditions similar to those used for cell fusion, the virus-erythrocyte mixtures were lightly fixed with 1 ml of 0.25% glutaraldehyde, and then treated first with anti-PR8 hyperimmune rabbit serum and then with the ferritin-conjugated goat IgG fraction against rabbit IgG (Cappel Labl., Pa.). Then the cells were fixed, embedded, sectioned, and observed in a Hitachi 11DS electron microscope as reported previously (Yasuda et al., 1982).

RESULTS

1. *Differential inactivation of influenza virus hemolytic and neuraminidase activities by heat treatment*

Figure 1 shows the time courses of heat inactivation of HA, HL and NA of influenza virus of several strains. At certain temperatures, (56 C for PR8; 55 C for Adachi and Tokyo), HA and HL of the influenza viruses were preserved but their NA was largely inactivated by heat-treatment for 30 min. When the temperature was raised, HA and HL were inactivated similarly (58 C for PR8; 50 C for FM1) and NA was inactivated much more rapidly. Similar tendencies were observed in all the other influenza viruses tested (WSN,

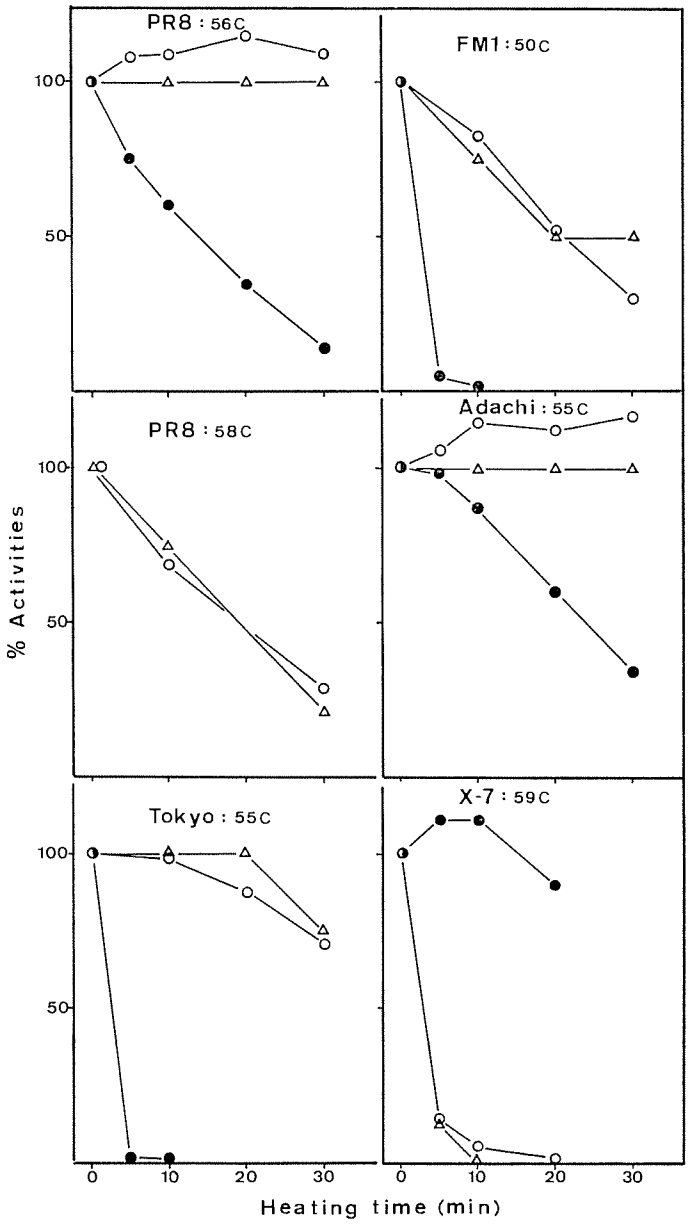

FIGURE 1. Heat-inactivation of influenza virus hemolytic (○), hemagglutination (△), and neuraminidase (●) activities. Strain names and inactivation temperatures are shown on panels.

Partially purified virions in isotonic saline containing 0.1% bovine serum albumin were heated at the indicated temperatures, and aliquots were taken at the times indicated for assays. Original HA titers: PR8, 15,000 HA/ml; FM1, 5,000 HA/ml; Adachi, 20,000 HA/ml; Tokyo, 20,000 HA/ml; X-7, 5,000 HA/ml.

Kum 1, Kum 2, Kum 3, BKK, B/Gifu, and B/Sing.) except the X-7 recombinant strain (Kilbourne et al., 1968) from the parents NWS (H0N1) and RI/5$^+$ (H2N2), in which NA was more heat-resistant than HA or HL.

Figure 2 shows the time courses of heat-inactivation of HA, HL, and NA of three viruses of the paramyxogroup. HL was inactivated firstly and HA and NA later. When the temperature was shifted up to above that at which HA was preserved, HA and NA were inactivated almost simultaneously (mumps virus,

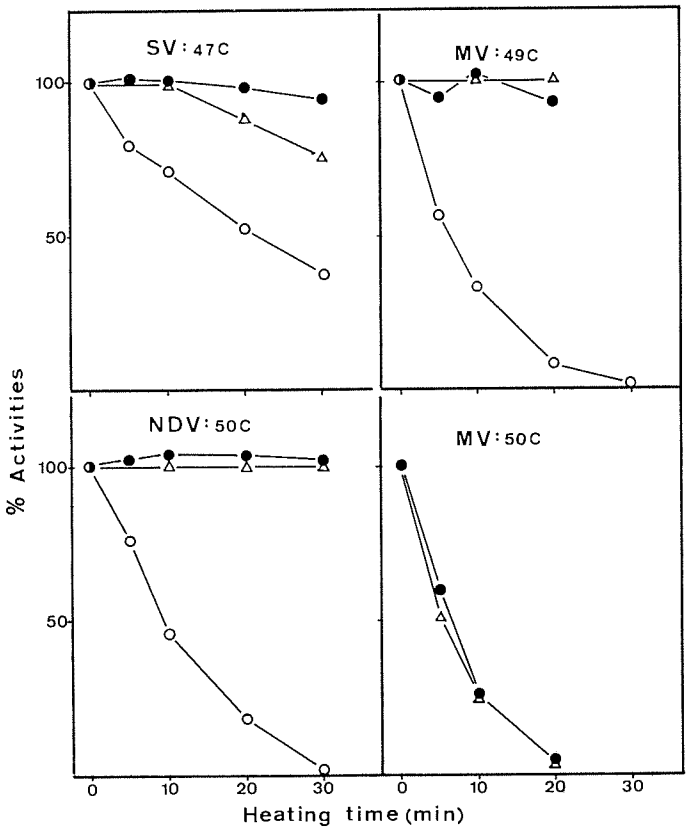

FIGURE 2. Heat-inactivation of paramyxovirus hemolytic (○), hemagglutination (△), and neuraminidase (●) activities. Virus names and inactivation temperatures are shown in panels.

Original HA titers: Sendai virus (SV), 16,000 HA/ml; NDV, 16,000 HA/ml; Mumps virus (MV), 10,000 HA/ml.

50 C) Similar tendencies were observed in the heat-inactivation of Sendai virus (50 C) and NDV (53 C). The finding that in paramyxovirus HL was heat-inactivated independently of HA and NA could be explained by the fact that HL was associated with fusion protein but not HN (hemagglutinin and neuraminidase) protein (Homma and Ohuchi, 1973; Scheid and Choppin, 1974). The present finding on the heat inactivation of influenza virus HL raises the possibility that the HL activity was not dependent on NA activity.

When WSN or BKK was heated at 55 C for 30 min, which resulted in almost complete inactivation of NA without inactivation of HA or HL, the infectivities of these strains found to be inactivated by an order of 10^{-5}.

2. *Inhibition of influenza virus HL by anti-HA and anti-NA sera*

Next we examined the inhibitions of influenza virus HL by anti-HA or anti-NA goat sera. Table 4 shows the results. Most inhibition was observed in homologous combinations of HA subtype between virus and antiserum. The most effective inhibitions were seen between virus and antiserum directed to the homologous hemagglutinin. When a virus of the same HA subtype isolated in a different epidemic was treated with the anti-HA serum, less inhibition was observed.

Since specific inhibitions of NA activity were seen only between X-7 or Kum 2 and anti-N2 serum among the combinations of viruses and anti-NA sera used (Table 3), we examined the HL in these combinations, but found negli-

TABLE 4. *Inhibition of influenza virus hemolysis by antisera against H0, H1, H2, H3, HA/B, and N1, N2, N/B: HLI titers*[a]

Virus strain	Antiserum							
	anti-H0 (PR8)[b]	anti-H1 (FM1)	anti-H2 (Sing. #)	anti-H3 (Aichi)	anti-HA/B (Lee)	anti-N1 (H_{eq} N1 ##)	anti-N2 (Sing. #)	anti-NA/B (Lee)
PR8 (H0N1)	68	0[c]	0	0	0	0	0	0
WSN (H0N1)	34	0	0	0	0	0	0	0
X-7 (H0N2)	20	0	0	0	0	0	0	0
FM1 (H1N1)	0	>80	0	0	0	0	0	0
Kum 2 (H2N2)	0	0	80	0	0	0	10	0
Aichi (H3N2)	0	0	0	>80	0	0	0	0
Tokyo (H3N2)	0	0	0	30	0	18	0	0
Kum 3 (H3N2)	0	0	0	10	0	12	0	0
BKK (H3N2)	0	0	0	0	0	0	0	0

[a] See "Materials and Methods".
[b] Parenthesis means the parent strain from which the immunogen was derived.
[c] Less than 10.
A/Singapore/1/57 (H2N2), ## A/Eq/Prague/1/56 (H_{eq})·NJ/8/76 (N1).

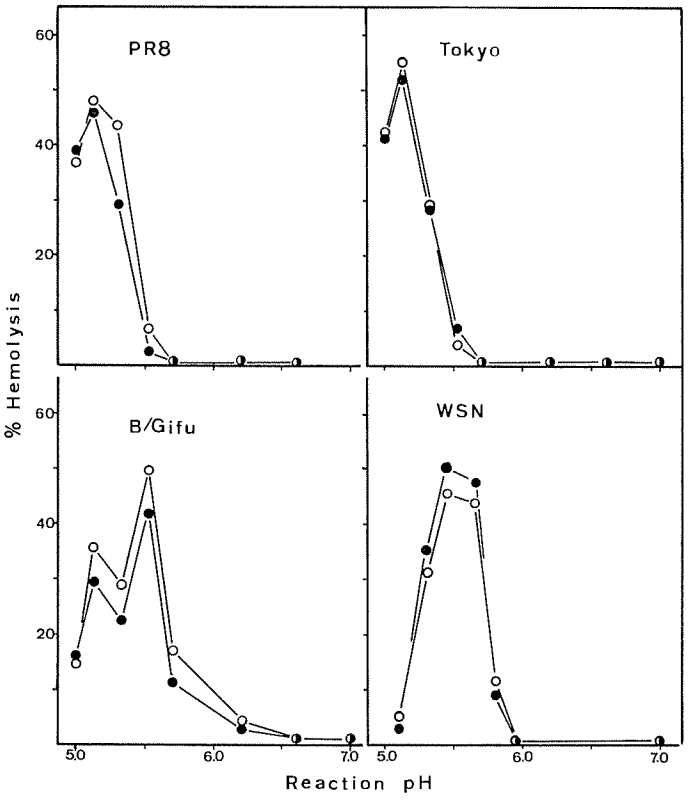

FIGURE 3. Optimal pH ranges for hemolysis by untreated (●) and heated (○) viruses. PR8, Tokyo, B/Gifu, and WSN strains were heated at 57 C, 55 C, 60 C and 55 C, respectively, for 30 min. Viruses with the HA titers described in Fig. 1 were used.

gible or slight inhibition of HL (Table 4). Some unknown inhibition of the HL was seen between some H3 strains (Tokyo and Kum 3) and anti-recombinant serum (A/Eq/Prague/1/56(Heq)–HJ/8/76(N1).

HL of influenza B type strains B/Gifu and B/Sing was found to be inhibited by all the antisera tested, and even by normal sera, and therefore this HL seemed to be largely affected by some serum proteins.

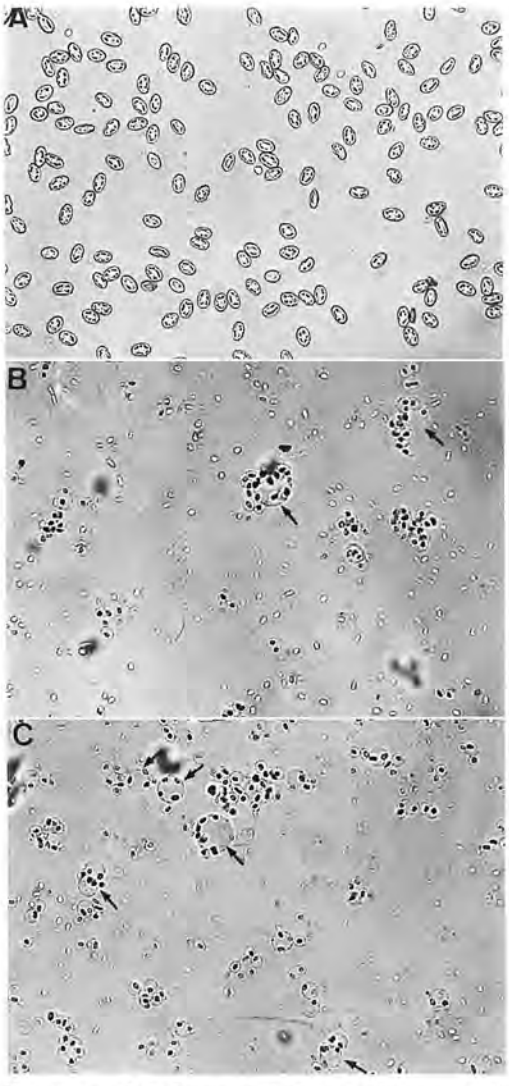

FIGURE 4. Cell fusion of chicken erythrocytes with influenza viruses. A. Control (no virus). B. Cell fusion with untreated PR8. C. Cell fusion with heated (57 C for 30 min) PR8. ×400.

Arrows indicate big polynuclear cells. 50 μl of PR8 (16,000 HA/ml in isotonic saline) was incubated with 100 μl of 2% chicken erythrocytes in acetate buffered saline (pH 5.3) at 37 C for 60 min.

3. *Some properties of influenza viruses with heat-inactivated neuraminidase*

1) pH range

The optimal pH ranges for hemolysis by the native influenza virus strains shown in Table 1 were examined. Results showed the presence of two types of pH range in the strains; one was a narrow pH range, where the hemolysis abruptly decreased at pH 5.5 (for PR8, FM1, Aichi, Tokyo, Kum 3, and BKK), and the other a wider range where hemolysis

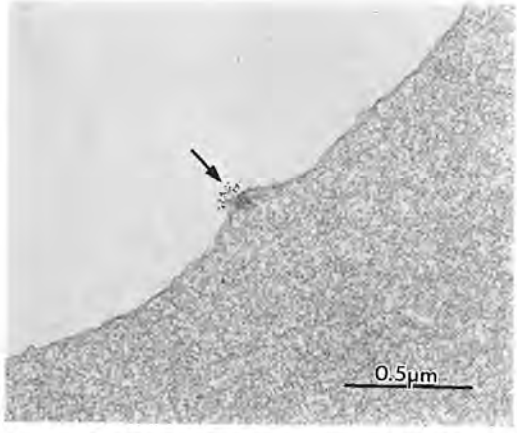

FIGURE 5. Envelope fusion of heated WSN with human erythrocytes observed by indirect immuno-electron microscopy.

Mixtures of heated (55 C for 30 min) WSN and human erythrocytes after incubation at 37 C for 10 min were firstly fixed with 0.25% glutaraldehyde, and then reacted with anti-PR8 serum, followed by treatment with ferritin-conjugated anti-rabbit IgG goat IgG fraction. Thereafter, the cells were fixed with Karnovsky's fixative and 2% OsO_4, and embedded in epoxy resin. Sections were stained with uranyl acetate and lead citrate and observed.

The arrow indicates a fused virion.

was seen at pH 5.0–5.8 (for WSN, Adachi, Kum 2, B/Gifu, and B/Sing.). Typical patterns are shown in Fig. 3. The pH ranges of heated viruses were almost the same as that of untreated viruses, as shown in Fig. 3.

This strain difference in the pH range did not affect the heat stability, or strength of the HL.

2) Cell fusion and envelope fusion

Figure 4 demonstrates cell fusions by native and heated (56 C for 30 min) PR8 of chicken erythrocytes. The heated virus induced as much cell fusion as the untreated virus.

Figure 5 is an indirect immunoelectron micrograph of the interaction of heated WSN (55 C for 30 min) and human erythrocytes observed after incubation at 37 C for 10 min. Ferritins, which indicate the localization of the virus antigens, are confined to an oval part of the erythrocyte membrane, suggesting the occurrence of envelope fusion.

These findings suggest that neither cell fusion nor envelope fusion depended on NA activity.

4. *Hemolytic activity of influenza virus grown in tissue culture*

To examine the effect of the host cells in which influenza virus was grown on the hemolytic activity, we prepared MDCK cell-grown WSN, partially purified it, and activated the virus with trypsin.

HL activity of the WSN strain (10,000 HA/ml) was found to be manifested best by treatment with a final concentration of 0.012% trypsin at 37 C for 10 min. This activated HL was compared with HL of egg-grown WSN with regard to its range, heat stability, and antiserum susceptibility. No significant difference was found between the two. Thus, these properties of HL activity of WSN seem to be independent on the host cells in which the virus is grown, and to be determined genetically.

DISCUSSION

The present experiments showed that influenza virus with heat-inactivated NA activity had hemolytic activity and was able to induce cell fusion and envelope fusion. If penetration of influenza virus is mediated by envelope fusion at low pH, this finding is consistent with earlier reports that influenza virus NA activity is not involved in the early steps of virus multiplication (its real role is to prevent virus aggregation) (Bucher and Palese, 1975): anti-NA antibodies did not affect the infectivity (Jahiel and Kilbourne, 1966; Kilbourne et al., 1968; Seto and Rott, 1966; Webster and Laver, 1967), and influenza viruses with heat-inactivated NA penetrated into cell (Fazekas de St. Groth, 1948), interfered with the replication of live virus (Isaacs and Edney, 1950), and took part in recombination with other strains (Burnet and Lind, 1954). Now, we propose that influenza virus NA activity does not have a role in the step of envelope fusion in more specific terms, instead of in general terms of virus penetration.

The present finding that NA activity probably did not have a role in influenza virus hemolysis is in contrast to the report by Huang et al. (1980a) that neuraminidase has a positive role in the fusion of influenza virosomes with cell membranes at neutral pH.

The present finding that influenza virus HL was heat-inactivated in the same way as hemagglutination, and that MDCK cell-grown WSN with possible precursor hemagglutinin (HA_0) had negligible HL support the hypothesis that the HL activity is associated with the hemagglutinin protein (Maeda et al., 1981).

There are similarities between hemolysis and fusion of influenza and togaviruses; 1) HL activity was associated with the hemagglutinin protein (Maeda et al., 1981; Yamamoto et al., 1981), and 2) HL and fusions were induced at low pH (Maeda and Ohnishi, 1980; Huang et al., 1981; Lenard and Miller, 1981; Karabatsos, 1963, 1965; Väänänen and Kääriäinen, 1979, 1980; Väänänen et al., 1981; Helenius

et al., 1980; White et al., 1980). The togaviruses were shown to penetrate into the cytoplasm by envelope fusion of virus to endocytotic vesicular membranes at low pH (Helenius et al., 1980; Marsh and Helenius, 1980; White et al., 1980). Therefore, influenza viruses probably penetrate into the cytoplasm by a mechanism similar to that of envelope fusion.

The infectivity was inactivated 10^{-5} by heat treatment that resulted in negligible NA activity, but did not affect HA. This type of heated virus fuse with cell membranes, and did not affect functional nucleic acids, which were postulated by the occurrences of interference and recombination (Isaacs and Edney, 1950; Burnet and Lind, 1954). Therefore, inactivation of the infectivity might be due to that of some other factors than viral RNA, such as transcriptase complex.

It is uncertain whether the inhibition of HL by anti-HA sera was primarily due to HI antibodies, or to HLI antibodies, which were assumed to be evoked independently of HI antibody, besides HI antibodies. Recently, Kimura et al. (1981) reported separation of HI antibodies with low HLI and low neutralization capacities from HLI antibodies with high neutralization but low HI capacities from among monoclonal antibodies directed to Japanese encephalitis virus. However, at present no such information is available on anti-influenza virus monoclonal antibodies.

NAs of influenza virus of the same NA subtype were frequently serologically different from each other (Shortridge et al., 1979; Chatiyanonda et al., 1981). In the present NI test (Table 3), anti-N2 (Sing.) inhibited the NA of only two of the 6 N2 strains tested. The slight inhibition of NA of Kum 2 by anti-N2 serum might be due to a steric effect of the NI antibodies. Since the anti-N1 serum used was an anti-recombinant one, and it contained anti-HA^1 as well as anti-N1 antibodies, it might cause unexpected and complex reactions inhibiting HL of some H3N2 strains.

We think that influenza viruses with heat-inactivated NA have some advantages for use in studies on envelope fusion or the behavior of fused virus antigens, because it can be studied under the circumstance free from the effects by NA activity.

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