Developmental Sequence and Intracellular Sites of Synthesis of Three Structural Protein Antigens of Influenza A₂ Virus

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Specific antisera for hemagglutinin (HA) and neuraminidase antigens of influenza A₂ virus (A₂E) were produced through the segregation of the two proteins in reciprocal viral recombinants of A₂E and A₀e viruses. Gamma globulin fractions of these specific antisera and of antiserum specific for the nucleoprotein (NP) antigen of A₀e virus were conjugated with fluorescein isothiocyanate and employed to follow the synthesis of the three structural proteins in clone 1-5C-4 human aneuploid cells, with parallel measurement of serological and biological activity of the antigens by other techniques. In this system, NP antigen appeared first (at 3 hr) in the cell nucleus, whereas HA and neuraminidase appeared coincidentally, at 4 hr after infection, in the cytoplasm. The initial detectability of biological or complement-fixing activity of the proteins coincided with their demonstrability as stainable antigens. Late in infection, all three antigens were detected at the cell surface. Antibody specific for HA partially blocked the intracellular staining of neuraminidase and inhibited the enzymatic activity of both extracted and intact extracellular virus. These observations suggest the close intracytoplasmic proximity of the two envelope antigens and perhaps their initial association in a larger protein.

It is now well established that influenza viruses contain at least three virus-coded antigenically distinguishable structural proteins: a nucleoprotein (NP), hemagglutinin (HA), and neuraminidase. Studies undertaken prior to the recognition that the neuraminidase is a discretely reactive, dissociable antigenic entity on the surface of the virus particle (12, 25) have followed, with fluorescein-conjugated antibody, the intracellular development of "viral" (V) and "S," "g," or NP antigen (1, 4, 13). In retrospect, it is clear that preparations of so-called anti-V antibody must have been mixtures of antibodies to both HA and neuraminidase proteins and consequently that the site and sequence of formation of the two antigens were not distinguished. It is probable that even antiserum to ether-split virus "HA" contained anti-neuraminidase antibodies as virus so disrupted has both HA and enzyme activity (2).

The segregation through genetic recombination of HA and neuraminidase (6, 8, 9) has greatly facilitated their physicochemical separation (12) and the production of antiserum specific for the

stable neuraminidase (7, 21) of influenza A₂ strains. Although the instability of the HA with sodium dodecyl sulfate disruption of virus has thus far precluded the production of antibody to the isolated protein, the availability of recombinant pairs reciprocal with respect to HA and neuraminidase has provided us with antisera monospecific in anti-HA (i.e., reacting only with HA) with appropriately chosen viruses.

The present paper describes the developmental sequence and intracellular sites of synthesis of NP, HA, and neuraminidase of a strain of influenza A₂ virus, as monitored with monospecific antisera with the immunofluorescence technique and with parallel assay of the biological activity of these proteins.

MATERIALS AND METHODS

Cell cultures. Clone 1-5C-4 derived from the Wong-Kilbourne variant of the Chang human aneuploid conjunctival cell line was used throughout the present study (24, 26). Cells were grown in medium 199 containing 10% fetal calf serum and were incubated at 36 C in a humidified atmosphere containing about 3% CO₂. Medium 199 with 0.1% bovine albumin was

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used as maintenance medium. Details of culture methods and media have been described (24).

Viruses. Influenza viruses A₀/NWS and A₂/RI/5⁺ (monkey kidney-adapted) and recombinants X-7, X-7(F1), and X-9 have been employed in earlier studies described elsewhere (6, 8, 12). Antigenic relationships among viruses employed in the present study are summarized in Table 1. [For clarity, viruses will be identified hereafter by symbolic designations indicating their surface (HA and neuraminidase) antigens.] Seed viruses were prepared by intra-allantoic inoculation of 10- to 11-day-old chick embryos and were stored at -90 C.

Immune sera. Virus materials employed for the preparation of antiserum were grown in all instances in the allantoic cavity of 10- to 11-day-old chick embryos. X-7, X-9, and NWS were purified by adsorption-elution on chick erythrocytes, followed by two cycles of differential centrifugation and sedimentation through a sucrose gradient. In the case of X-9 and NWS, elution required the addition of *Vibrio cholerae* neuraminidase (receptor-destroying enzyme).

The purified viruses (10⁴ HA units) were injected into the marginal ear veins of adult rabbits which were re-injected with the same amount of antigen after 40 days. Blood was obtained from all rabbits by cardiac puncture before immunization and at 7 and 14 days after the second injection. Specific antiserum (R-296) against the A₂/RI/5⁺ enzyme (E) was prepared in the rabbit by immunization with the enzyme protein of A₂/RI/5⁺, isolated after separation by electrophoresis of SDS-disrupted X-7(F1) on cellulose acetate strips. The procedure of immunization and extensive studies of this antiserum have been described elsewhere (7). For complement-fixation (CF) tests for NP antigen, antiserum against the S (NP) antigen of influenza A was purchased from Microbiological Associates (CF titer 1:32). All antisera were stored at -20 C and heated to 56 C for 30 min before testing.

TABLE 1. *Antigenic constitution of influenza viruses employed in this study*

Virus	Antigens			Symbolic designation
	Nucleo-protein ^a	Hemagglutinin	Neuraminidase	
A ₀ /NWS	A ₀	A ₀	e	A ₀ e
A ₂ /RI/5 ⁺	A ₂	A ₂	E	A ₂ E
X-7	A ₀	A ₀	E	A ₀ E
X-7(F1)	A ₀	A ₀	E(2)	A ₀ E(2)
X-9	A(?)	A ₂	e	A ₂ e

^aNucleoproteins were indistinguishable in complement-fixation reactions. A₀ (NWS-like) nucleoprotein antigens were distinguishable from A₂ (RI/5-like) nucleoprotein antigens by peptide mapping (12). Nucleoprotein of X-9 was not mapped.

^bX-7 (F1) has twice the amount of neuraminidase as X-7 (25).

Preparation of NP antigen. The method followed was similar to that described by Kirber and Henle (10). Eleven-day-old chick embryos were inoculated intra-allantoically with 10⁸ EID₅₀ of A₀/NWS virus and incubated at 36 C for 24 hr. Homogenates of infected allantoic membranes were centrifuged at 2,000 rev/min for 15 min to remove larger particles. Virus particles were removed by absorption on chick erythrocytes and centrifugation at 26,360 × g for 1 hr. After further centrifugation at 80,730 × g for 2 hr, the resulting sediment was suspended in phosphate-buffered saline (PBS) so as to contain a CF titer of 1:40 against 4 units of NP antiserum. This NP antigen preparation was free from HA as demonstrated by testing with chick erythrocytes.

CF test. CF tests were done by a modification of Kilmer's method. Briefly, 0.1 ml of a twofold serial dilution of antigen, 0.1 ml of 4 units of antiserum, and 0.2 ml of 2 units of complement were mixed and allowed to stand for 1 hr in a water bath at 37 C. Then 0.2 ml of 2.5% sheep red cells, sensitized with 3 units of hemolysin, was added and allowed to stand for 30 min in a water bath at 37 C. The CF titer was expressed as the highest dilution of antigen exhibiting more than 75% fixation of complement.

Hemagglutination titrations. Hemagglutination was carried out in tubes with the samples serially diluted twofold in 0.4 ml of 0.01 M PBS. An equal volume of 0.5% chicken erythrocytes was added to each tube, and the pattern was read after incubation for 30 min at room temperature.

Hemagglutination-inhibition (HAI) tests. Twofold serial dilutions of serum were incubated with 8 HA units of virus for 1 hr at room temperature before the addition of chicken erythrocytes. The titer was expressed as the highest dilution of serum which caused complete inhibition of hemagglutination. Nonspecific inhibitors were inactivated by treatment of serum with 3 volumes of 0.01 M KIO₄ at room temperature for 30 min. Periodate was then neutralized by the addition of an equal volume of 10% glucose in saline.

Infectivity titrations. Infectivity titrations of A₂/RI/5⁺ were carried out by intra-allantoic inoculation of four or five chick embryos with 0.1 ml of each of 10-fold serial dilutions of test materials in maintenance medium. Allantoic fluids were tested for HA after 2 days of incubation at 36 C. The 50% infectivity end points (EID₅₀) were calculated by the method of Reed and Muench. Infectivity titration of influenza viruses other than A₂/RI/5⁺ was done by the plaque method in clone 1-5C-4 cells (24).

Neuraminidase assay and enzyme inhibition test. Enzyme assay and enzyme inhibition tests were performed with a fetuin substrate by a modification of Warren's thiobarbituric acid method as described previously (12). One unit of neuraminidase was defined as the amount of enzyme required to yield sufficient N-acetylneuraminic acid (NANA) to produce an optical density (OD) of 0.1 at 549 nm, with an enzyme-substrate (fetuin) reaction time of 30 min. Enzyme activity was measured by OD readings taken from the part of the slope where they vary linearly with the enzyme concentration. Four to 6 units of enzyme was employed in enzyme-inhibition tests.

Fluorescent-antibody staining. A gamma-globulin fraction of antiserum was prepared by precipitation with cold, one third-saturated ammonium sulfate, then conjugated with fluorescein isothiocyanate by the method of Shimojo et al. (23), and immediately passed through a Sephadex G50 gel column. The conjugate was further purified by passage through a diethylaminoethyl cellulose column and absorption with acetone-precipitated human liver powder to reduce nonspecific staining.

Absorption of fluorescein-conjugated antisera and dissociation of antigen-antibody complexes. Fluorescein-conjugated antiserum against X-7 (A₀E) virus was mixed with concentrated NWS (A₀E) particles, and the mixture was allowed to stand for 1 hr at room temperature (Table 2). The A₀-antibody complex was removed by centrifugation at 80,730 × *g* for 1 hr. The supernatant fluid was reabsorbed with NWS particles in a similar way. Further absorption of the conjugate with NP antigen was carried out in the same way as described above, except for centrifugation at 80,730 × *g* for 2 hr. The resulting supernatant fluid had a HAI titer of less than 1:8 against either NWS (A₀E) and X-9 (A₀E) and a 50% enzyme inhibition titer of between 1:640 and 1:2,560 against the X-7(F1) (E) enzyme.

The A₀ antibody complex and NP antigen-antibody complex were each resuspended in 0.1 M glycine hydrochloride NaCl buffer (pH 2.6) containing 1% (v/v) inactivated normal rabbit serum for 1 hr at room temperature. NWS particles and NP antigen were removed by centrifugation at 35,000 rev/min for 1 and 2 hr, respectively. The respective supernatant fluids were adjusted to pH 7.0 by the addition of 1.0 M sodium phosphate buffer and then were dialyzed overnight against PBS.

NP antibody present in fluorescein-conjugated antiserum against A₂e and A₀e viruses was also removed by absorption with NP antigen by the method described above. After complete removal of NP antibody, conjugated anti-A₂e serum and anti-A₀e serum had HAI titers of 1:1,024 against the respective homologous virus and less than 1:8 against the heterologous virus.

Fluorescein-conjugated antiserum against the isolated A₂ enzyme had a 50% enzyme inhibition titer of

1:2,560 against X-7 (F1) virus-associated enzyme. All conjugates were stored at -20 C.

Preparation of infected cells for staining. The direct fluorescent-antibody technique was employed. Control and infected cell monolayers on cover slips in Leighton tubes were gently washed with PBS, fixed with cold acetone for 5 min, and then washed again with PBS after the acetone had evaporated. These cover slip cultures were stained with the conjugate in a water-saturated chamber at 36 C for 1 hr or at 4 C overnight. They were then rinsed in three changes of PBS to remove the remaining nonbound fluorescent antibody and mounted on a slide glass with 90% glycerol in PBS.

Serum blocking test. A serum blocking test was done to confirm the specificity of staining. Unconjugated antiserum was placed on a cover slip in a humidified chamber at 36 C for 1 hr.

After removal of the antiserum, the cover slip was rinsed in PBS and stained with fluorescein-conjugated antiserum.

Fluorescence microscopy. Preparations were examined with a Zeiss fluorescence microscope equipped with an Osram HBO-200 mercury vapor lamp, a GB 12 exciting filter, and a no. 47 barrier filter.

One-step growth experiment. Clone 1-5C-4 cell monolayers in plastic petri dishes were washed with PBS and then infected with A₂e at an input multiplicity of approximately 20 EID₅₀ per cell. After 1 hr of incubation, monolayers were washed with PBS twice, further incubated with 2 ml of a 1:100 dilution of A₂e serum for 15 min, and washed again three times with PBS. A 2-ml amount of maintenance medium was added to each petri dish, and the cultures were further incubated in a CO₂ incubator at 36 C. At various intervals after infection, cells and culture fluids were harvested separately from two petri dishes and pooled. Cell suspensions in 2 ml of maintenance medium were subjected to three cycles of freezing and thawing and centrifugation at low speed. The resulting supernatant fluids and culture fluids were assayed separately for intracellular and extracellular biological activity, respectively.

TABLE 2. *Preparation of conjugated antisera*^a

Antiserum	Immunogen	Antiserum absorbed with	Antiserum specific for
Anti-E	A ₀ E virus	NP antigen and A ₀ e	A ₂ neuraminidase (E)
Anti-E ^b	E ^c		A ₂ neuraminidase (E)
Anti-NP	A ₀ E virus	NP antigen (then dissociation of NP-antibody complex)	Nucleoprotein (NP)
Anti-A ₀	A ₀ E virus	A ₀ e (then dissociation of A ₀ -antibody complex)	A ₀ hemagglutinin (A ₀)
Anti-A ₂ e	A ₂ e virus	NP antigen	A ₂ hemagglutinin A ₀ neuraminidase (e)

^aAll antisera were conjugated with fluorescein isothiocyanate before absorption.

^bR-296 (see Materials and Methods).

^cIsolated enzyme from A₀E(2).

RESULTS

Tests for specificity of fluorescein-conjugated antisera. Earlier studies have demonstrated that, under conditions of multiplication leading to formation of complete influenza virus, NP antigen appears in the nucleus in early stages of infection and later in the cytoplasm, whereas "virus" ("V"; HA plus neuraminidase) antigen formation is restricted to the cytoplasm. On the basis of these findings, cover slips 4 and 17 hr postinfection were used for nuclear staining of NP antigen and cytoplasmic staining of viral envelope antigens, respectively. Cell monolayers on cover slips were infected with A₂E, A₀E, A₀E(2), A₂e, or A₀e viruses at high multiplicity. Cover slips were taken out 4 and 17 hr postinfection and stained with conjugated antisera (Table 3).

Conjugated neuraminidase antiserum, whether obtained after absorption of conjugated A₀E antiserum with NP antigen and A₀e virus or produced by immunization with isolated enzyme from A₀E(2) virus, did not produce nuclear fluorescence in infected cells 4 hr postinfection or cytoplasmic fluorescence in A₂e- or A₀e-infected cells 17 hr postinfection. These observations and the absence of HAI activity against A₀e and A₂e and the presence of high enzyme inhibition activity in these conjugated "antineuraminidase"

sera demonstrated their monospecificity for neuraminidase antigen (E) in the systems studied. To confirm further the specificity of these antisera in the staining of enzyme antigen, serum blocking tests were carried out. Table 4 shows that both anti-enzyme sera (anti-E and absorbed A₀E serum) completely blocked the production of cytoplasmic fluorescence 17 hr postinfection in A₂E-infected cells when staining was attempted with conjugated neuraminidase antiserum, whereas anti-NP serum (CF titer of 1:32) did not block it. However, undiluted A₂e antiserum (HAI titer of 1:2,048) caused marked reduction in intensity of fluorescence of the enzyme (E) antigen. As discussed later in this paper, it seems most likely that this phenomenon was not due to a blocking of staining by contaminating antibody to the A₂ (E) enzyme but rather to steric hindrance by the anti-HA antibody of A₂e antiserum with the intracellular combination of antineuraminidase (anti-E) with enzyme antigen (E). It was concluded that the cytoplasmic fluorescence in A₂E-, A₀E-, A₀E(2)- or X-7(F1)-infected cells stained with conjugated antineuraminidase antiserum represents specific staining of the enzyme antigen (E) of A₂E.

Conjugated NP antibody and conjugated anti-A₀ (HA) antibody prepared by dissociation of

TABLE 3. Specificity of fluorescein isothiocyanate-conjugated antisera in immunofluorescence^a

Cells infected with	Time post-infection	Antiserum ^b											
		Anti-E (a)		Anti-E(b)		Anti-NP		Anti-A ₀		Anti-A ₂ e		Anti-A ₀ e	
		NF	CF	NF	CF	NF	CF	NF	CF	NF	CF	NF	CF
A ₂ E	4 hr	0	+	—	—	+	0	—	—	0	+	0	0
	17	0	++	0	++	0	+	0	0	0	++	0	0
A ₀ E	4	—	—	0 ^c	0 ^c	+	0	—	—	0	0	—	—
	17	0	+	—	—	—	—	0	++	0	0	0	++
A ₀ E(2)	4	—	—	—	—	—	—	—	—	0	0	—	—
	17	0	+	—	—	—	—	0	++	0	0	0	++
A ₀ e	4	—	—	—	—	+	0	—	—	—	—	—	—
	17	0	0	0	0	0	+	0	++	0	0	0	++
A ₂ e	4	0	0	—	—	+	0	—	—	—	—	0	0
	17	0	0	0	0	0	+	0	0	0	++	0	0
No virus		0	0	0	0	—	—	—	—	0	0	0	0

^aAbbreviations: NF, nuclear fluorescence; CF, cytoplasmic fluorescence; 0, no fluorescence; +, distinct fluorescence; ++, intense fluorescence; —, not tested; (a), immunogen was intact virus (A₀E); (b), immunogen was isolated enzyme (E).

^bResults at optimal dilution (0 to 8).

^cThree hours postinfection.

TABLE 4. *Blocking of specific fluorescence by unconjugated antisera*

Cells infected with	Time post-infection	Stained with	Blocking antiserum	Specific fluorescence ^a	
				NF	CF
A ₂ E	4	Conjugated anti-NP	Non-treated	++	0
			Anti-A ₂ E (1:10) ^b	++	0
			Anti-NP (undiluted)	±	0
A ₂ E	17	Conjugated anti-A ₂ E	Nontreated	0	++
			Anti-NP (undiluted)	0	++
			Anti-A ₀ E (1:4)	0	++
			Anti-A ₂ E (1:8)	0	±
A ₂ E	17	Conjugated anti-E	Nontreated	0	++
			Anti-NP (undiluted)	0	++
			Anti-E (1:4)	0	0
			Anti-A ₂ E (1:4)	0	0
			Anti-A ₂ E (1:8)	0	+
			Anti-A ₂ E (undiluted)	0	±

^a See footnote a to Table 3. Equivocal fluorescence, ±.^b Antiserum dilution.

antigen-antibody complexes were also tested for their specificity of staining (Table 3 and Materials and Methods). With dissociated conjugated NP antibody, fluorescence was detected at 4 hr post-infection in the nuclear region of cells infected with either A₀E or A₂E virus. This cross-reactivity was expected (13). In the serum blocking test (Table 4), the intensity of nuclear fluorescence was markedly reduced by unconjugated NP antiserum (undiluted) but was not changed by anti-A₂E (anti-A₂ HA) serum diluted 1:10. It was shown that dissociated conjugated anti-NP antibody had an HAI titer of less than 1:10 against A₂E and of 1:20 against A₀E. Therefore, in subsequent experiments, dissociated conjugated NP antibody was used for staining of NP antigen in A₂E-infected cells.

On the other hand, dissociated conjugated A₀ (HA) antibody had an HAI titer of 1:5,120 against A₀E and an HAI titer of 1:160 against A₂E. A 1:16 dilution of this dissociated antibody produced cytoplasmic fluorescence in A₀E- or A₂E-infected cells 17 hr postinfection. At a 1:2 dilution, the antibody showed weak cytoplasmic fluorescence in some A₂E- or A₂E-infected cells 17 hr postinfection, probably because of slight cross-reactivity between the hemagglutinins of A₂ and A₀ strains (8). The absence of fluorescence of A₂E-infected cells illustrated in Table 3 shows that the conjugated A₀ anti-HA antibody was not con-

taminated with either conjugated NP antibody or anti-neuraminidase.

Conjugated A₂E antiserum after absorption with NP antigen showed cytoplasmic fluorescence at dilutions as high as 1:8 in A₂E-infected cells 17 hr postinfection, whereas A₀E-, A₀E-, or A₀E(2)-infected cells were stained with A₀E-conjugated antiserum at comparable dilutions. At 1:2 dilutions, no nuclear fluorescence appeared in infected cells 4 hr after infection with either antiserum. In addition, Table 3 shows that neither A₂E-infected cells stained with conjugated A₀E antiserum or A₀E-infected cells stained with conjugated A₂E antiserum revealed any visible fluorescence related to the presence of the common neuraminidase enzyme (e) antigen in these two viruses. As remarked in previous studies (7, 19), the A₀/NWS neuraminidase (e) is unstable, not only with respect to its hydrolytic activity but also its immunogenicity. Indeed, it was determined that the plaque size reducing (PSR) titer of the conjugated A₂E antisera (a measure of their antineuraminidase activity; 6) was only 1:1,100 compared to the 1:25,600 PSR titer of the conjugated X-7 anti-E antiserum used in the present experiments. Table 4 shows that the cytoplasmic fluorescence in A₂E-infected cells stained with conjugated A₂E antiserum was markedly blocked by A₂E antiserum but not by NP antiserum and A₀E antiserum. Therefore, it is clear that the conjugated A₂E antiserum prepared was reactive only with the HA antigen of A₂E when A₂E-infected cells were stained with the conjugate. This conjugated A₂E antiserum was used for the staining of A₂E HA antigen in subsequent experiments. These studies of the specificity of immunofluorescent staining of HA and neuraminidase antigens within cells add further confirmation to the extensive evidence for segregation of these proteins in recombinant viruses adduced from studies of intact or disrupted viruses.

Study with monospecific fluorescent antibody of influenza A₂E virus multiplication in clone 1-5C-4 cells. The development of viral enzyme, HA, and NP antigen in A₂E-infected cells was investigated with the fluorescein-conjugated monospecific antisera described above.

Cell monolayers grown on cover slips in Leighton tubes were washed with maintenance medium and then infected with A₂E at an input multiplicity of approximately 100 EID₅₀ per cell. After 1 hr of adsorption, monolayers were washed with maintenance medium and further incubated with maintenance medium at 36 C. At appropriate intervals after infection, cover slips were removed, fixed, and stained with fluorescent antibody.

Development of NP antigen. The fluorescent

NP antigen was first detected in the nuclei of all cells 3 hr postinfection (Fig. 1). Thereafter, the intensity of the nuclear fluorescence increased, and fluorescent NP antigen began to spread out diffusely into the cytoplasm in the form of fine fluorescent granules 4.5 hr postinfection. With further incubation, the number of cells showing

nuclear staining decreased, and the fluorescent NP antigen was observed to be more widely distributed in the cytoplasm. Seventeen hours after infection, NP antigen was detected principally in the cytoplasm as a diffuse fluorescence, with stronger fluorescence at the cell surface.

Development of viral neuraminidase and HA

FIG. 1. *A₂E*-infected cells stained with fluorescein-conjugated NP antibody. (1a) At 2 hr postinfection, no fluorescence. (1b) At 3 hr postinfection; fluorescence is in the region of the nucleus. (1c) At 7 hr postinfection, fluorescence is widely distributed in the cytoplasm. Some cells have lost nuclear fluorescence. (1d) At 17 hr postinfection, fluorescence is principally in the cytoplasm, concentrated at cell margins. $\times 200$.

antigen. Both neuraminidase antigen (Fig. 2) and HA antigen (Fig. 3) were first detected in the perinuclear region of the cytoplasm 4 hr after infection. At this stage of infection, the fluorescence of both antigens was frequently found in especially high concentration in a localized area of the cytoplasm near the nucleus. With further in-

cubation, the fluorescence of both antigens increased in intensity and was detected later at the cell margin. At 6.5 to 8 hr postinfection, both antigens were widely distributed in the cytoplasm of a large number of cells and, at 17 hr after infection, were demonstrable throughout the cytoplasm of all cells, with stronger fluorescence at the

FIG. 2. A₂E-infected cells stained with fluorescein-conjugated neuraminidase antibody. (2a) At 3 hr postinfection, no fluorescence. (2b) At 4 hr postinfection, cytoplasmic (perinuclear) fluorescence. Note strong fluorescence at one pole. (2c) At 6.5 hr postinfection, general cytoplasmic fluorescence. (2d) At 17 hr postinfection, increased cytoplasmic fluorescence. $\times 200$.

FIG. 3. *A₂E*-infected cells stained with fluorescein-conjugated HA antibody. (3a) At 3 hr postinfection, no fluorescence. (3b) At 4 hr postinfection, perinuclear, polar cytoplasmic fluorescence (as in Fig. 2b). (3c) At 6.5 hr postinfection, general cytoplasmic fluorescence. (3d) At 17 hr postinfection, increased cytoplasmic fluorescence concentrated at cell margins. $\times 200$.

cell surface. In contrast to the initial appearance of NP antigen in the nucleus, enzyme antigen and HA antigen were detected early only in the cytoplasm. No difference between enzyme antigen and HA antigen in localization or time of appearance was observed.

Correlation with biological activity: assay of

infectivity, HA, and CF-NP activity. A one-step growth curve experiment with *A₂E* in clone 1-5C-4 cells was carried out by using an input multiplicity of approximately 20 EID₅₀ per cell (Fig. 4). Intracellular CF-NP activity and intracellular HA were first detected 4 and 5 hr postinfection, respectively, and reached a maximum level 8 and

FIG. 4. One-step growth of A₂E in clone 1-5C-4 cells. The input multiplicity of infection (EID₅₀ per cell) was approximately 20. Symbols: ○, intracellular infectivity titer; ●, extracellular infectivity titer; △, intracellular HA titer; ▲, extracellular HA titer; ×, intracellular CF-S titer. Curves were fitted by eye to points indicated.

10.5 hr postinfection, respectively. Both intracellular and extracellular infectivity began to rise after a latent period of 6 hr and continued to increase exponentially for as long as 10.5 hr postinfection. Thereafter, intracellular infectivity declined while a gradual increase of extracellular infectivity continued. Extracellular HA was first detected 10.5 hr after infection and reached a maximum titer at 20 hr; its concentration was usually low compared with that of intracellular HA.

Assay of viral neuraminidase: effect of non-infected cell extracts on the assay of A₂E neuraminidase. Before the assay of viral neuraminidase in the cells after infection with A₂E, it was necessary first to examine the effect of noninfected cell extracts in the assay system. Extracts of noninfected cells were prepared in the same way as the infected cell extracts. Experimental design was similar to that used in prior studies in chick embryo chorioallantoic membranes (18). The reaction mixture was adjusted to pH 5.9 with 0.4 M sodium phosphate buffer and contained the components listed in a total volume of 0.4 ml. After incubation in a water bath at 37 C for 30 min, 0.2 ml of the reaction mixture was used for the assay of free NANA (see above). Noninfected cell extracts did not contain measurable quantities of free NANA or other color-forming substances. Neither detectable neuraminidase activity nor any demonstrable substrate for A₂E virus enzyme was present in the extract from noninfected cells. The enzyme activity of A₂E was slightly enhanced in the presence of noninfected cell extract.

Assay of intracellular A₂E neuraminidase activity. Assay of intracellular neuraminidase was carried out during a one-step growth curve experiment with A₂E in clone 1-5C-4 cells. A 0.2-ml amount of test materials diluted with saline was added to 0.1 ml of fetuin and adjusted to pH 5.9 with 0.1 ml of 0.4 M sodium phosphate buffer. After 30 min of incubation in a water bath at 37 C, 0.2 ml of the reaction mixtures was used for the assay of free NANA. OD was read at 549 nm against a blank tube containing fetuin plus saline. Assay of test materials without fetuin substrate was also carried out, when necessary, to correct the OD reading at 549 nm (OD_{549nm}) of NANA released in the above experiments. In the assay of test materials without fetuin substrate, the blanks did not exceed 0.008 OD_{549nm} in any case. After correction for the blanks, the amount of NANA released in test materials was proportional to the concentration of test material in all cases. The increase of intracellular viral neuraminidase activity in this experiment is shown in Fig. 5. The intracellular enzyme activity first appeared between 4 and 5 hr postinfection, continued to increase exponentially for as long as 8 hr, and reached a plateau at 10.5 hr. In a preliminary experiment, it had been found that the presence of low enzyme activity immediately after infection was related to the residual viral inoculum.

It is clear that the curve of the intracellular enzyme activity shown in Fig. 5 parallels the increase in intracellular HA demonstrated in Fig. 4. These results are consistent with the coincident develop-

FIG. 5. Intracellular synthesis of viral neuraminidase in clone 1-5C-4 cells infected with A₂E at an input multiplicity of approximately 20. Intracellular enzyme units were determined on the basis of OD_{549nm} readings taken from the slope where they vary linearly with the dilution of the test materials (see Fig. 5.) Symbols: ○, neuraminidase in A₂E-infected cells; ×, neuraminidase in A₂E-infected cells; ×, neuraminidase concentration of noninfected cells; ●, HA (from Fig. 4).

ment of enzyme antigen and HA antigen in A₂E-infected cells shown by fluorescent-antibody staining. In addition, both immunofluorescent studies and the one-step growth curve experiment indicated that the intracellular NP antigen appeared 1 hr earlier than either intracellular neuraminidase or HA in A₂E-infected cells.

Inhibition by specific antiserum of the activity of neuraminidase produced in A₂E-infected cells. The results of the one-step growth curve experiment and immunofluorescent studies indicate that the neuraminidase produced in A₂E-infected cells is viral. Inhibition of enzyme activity by monospecific antiserum provided additional evidence that the intracellular enzyme appearing after infection with A₂E corresponds, in fact, to the neuraminidase derived from A₂E.

The cell extract 17 hr after infection of monolayers with A₂E virus was incubated for 1 hr at room temperature with equal volumes of threefold serial dilutions of anti-enzyme serum (anti-E₂), A₂E antiserum (HAI titer of 1:2,560), A₂E antiserum (HAI titer of 1:2,048), or normal rabbit serum before assay of enzyme activity. The enzyme activity in the presence of antiserum was

compared with the activity in the presence of normal serum of equivalent dilutions.

As shown in Fig. 6, the intracellular enzyme activity 17 hr postinfection was inhibited by antiserum specific for the enzyme of A₂E (R-296) and, unexpectedly, to some extent by A₂E antiserum, but not by anti-A₀E. This inhibition of viral neuraminidase by antiserum which did not contain antibodies to A₂ neuraminidase was examined further.

Inhibition of viral neuraminidase activity by anti-HA antibody. It was shown that A₂E antiserum was inhibitory to the activity of enzyme from A₂E-infected cells and that, in a serum blocking test, unconjugated anti-A₂E blocked the staining of enzyme antigen in A₂E-infected cells with conjugated anti-enzyme serum. The antigenic structure of A₂E and the activity of A₂E antiserum suggested that antibody to the HA component of A₂E (A₂) may play an important role in these phenomena. To confirm this possibility, A₂E antiserum was tested at twofold serial dilutions for its ability to inhibit the neuraminidase activity of egg-grown A₂E, A₀E, and A₀E(2) virus particles, all of which possess the same A₂

FIG. 6. Inhibition of activity of neuraminidase produced in A₂E-infected cells by neuraminidase antiserum. Test material 17 hr postinfection (Fig. 4) was incubated with an equal volume of anti-enzyme serum (R-296; ○), anti-A₀E serum (×), anti-A₂E (anti-HA) serum (●), or normal rabbit serum diluted serially threefold. Neuraminidase activity in the presence of antiserum was compared with the activity in the presence of normal serum at equivalent dilutions.

FIG. 7. Inhibition of enzyme activity by anti-HA antibody. Preparations of A₂E (○), A₀E(2) (×), and A₀E (●) viruses were incubated with an equal volume of a twofold serial dilution of anti-A₂E serum (HAI titer of 1:2,048) at room temperature for 1 hr and then assayed for enzyme activity. Enzyme activity was also tested by preincubating each of the viruses with normal rabbit serum in a similar way and by comparison with the activity in the presence of anti-A₂E serum at equivalent dilutions.

enzyme (E) on their surface. As demonstrated in Fig. 7, A₂e antiserum did not inhibit the enzyme activity of A₀E and A₀E(2) virus particles, which share no envelope antigens with A₂e, whereas it was inhibitory to the enzyme activity of A₂E virus particles which share the same HA (A₂) with A₂e. It is probable that the inhibition of enzyme activity of A₂E by A₂e antiserum is due to steric hindrance of enzyme action by coverage of adjacent HA sites with anti-HA antibody.

DISCUSSION

In accord with earlier studies, NP antigen was first demonstrable in the cell nucleus and then appeared as granular masses within the cytoplasm, which became prominent at the cell borders later in infection as specific nuclear fluorescence became less marked. Not surprisingly, HA antigen (stained specifically in our studies for the first time) followed the pattern previously observed for V antigen maturation, i.e., initial detectability 1 hr later than NP antigen and appearance only in the cytoplasm. It is interesting that the timetable of antigen maturation in our studies of (human) influenza A₂ virus in a human aneuploid cell line corresponds exactly with Breitenfeld and Schafer's classical investigation of fowl plague virus in chick embryo fibroblasts (1), another species-homologous system. The slower appearance of antigens observed with another influenza A₂ strain in bovine embryo kidney cells (4) may have been virus- or host cell-determined, but, in any case, led to qualitatively similar observations.

Intracellular viral neuraminidase has not been demonstrated previously as stainable antigen; however, it has been detected as active enzyme 3 hr after infection in chorioallantoic membranes and monkey kidney cells, and, by extrapolation, its synthesis has been estimated to start as early as 1 to 2 hr after infection (17). In the present studies, the initial appearance of neuraminidase as antigen demonstrable by immunofluorescence (at 4 hr) coincided closely with its appearance as cell-associated active enzyme. The detectability of HA by immunofluorescence and biological activity was also coincident and is in agreement with the development of the viral neuraminidase. The initial demonstration of infectivity (at 6 hr) coincided with the demonstration of all three antigens at the cell margin at 6.5 to 7 hr.

Our data, based on fluorescent-antibody staining or measurement of viral activity in whole-cell extracts, do not exclude the possibility of initial intranuclear synthesis of either HA or neuraminidase, as the experiments of Scholtissek and his colleagues suggest (20). The demonstration of all three viral components at the cell surface in the

later stages of infection is in agreement with evidence that influenza virus is assembled at the cell surface (16).

Of special interest is the demonstration that specific anti-HA antibody not only blocked intracellular staining of antigenically heterotypic viral neuraminidase by conjugated antineuraminidase but also inhibited the enzymatic activity of intact particles of such virus. The latter instance is readily explained by steric hindrance (3, 22), based on evidence that the ratio of HA to neuraminidase on the virus surface is 2:1 or 4:1 (25), so that antibody to HA may nonspecifically block access of the hydrolytic site of the enzyme from its substrate. If similar factors operate intracellularly (but assuming blockage of the antigenically reactive rather than the enzymatically reactive site of the neuraminidase), then it follows that antigenically active HA and neuraminidase polypeptides are either closely associated spatially or may even exist initially as fragments of a larger protein, subject to later cleavage as seems to be the case with poliovirus (5). This postulation is belied, in part, by evidence that the major part of neuraminidase activity is first found free from HA activity in postmicrosomal "soluble" fraction of disrupted cells (17), but HA activity and antigenicity are, of course, not synonymous. Moreover, the same studies show that, after 4 hr, the soluble enzyme activity decreased to about 20% with a concomitant rise in heavier fractions (>100S).

Study of the influence of HA antibody on heterotypic cell-associated viral neuraminidase activity early in infection may be instructive in defining the state of the intracellular proteins.

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