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Antiviral Activity of Antiserum Specific for an Influenza Virus Neuraminidase

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Antiserum specific for influenza A_2 neuraminidase was produced by immunization of rabbits with the purified enzyme which had been isolated by electrophoresis from the proteins of a detergent-disrupted A_0A_2 influenza virus recombinant [X-7 (F1)]. This recombinant contained hemagglutinin of the A_0 subtype and A_2 neuraminidase. Antiserum to the isolated A_2 neuraminidase did not react in any of four serological tests with A_0 or A_2 subtype viruses that lacked the A_2 enzyme. In contrast, the antiserum inhibited the neuraminidase activity only of wild-type and recombinant viruses containing the A_2 enzyme, regardless of the nature of their hemagglutinin proteins. The antiserum caused hemagglutination-inhibition of some, but not all, viruses bearing the A_2 enzyme, and it reduced the plaque size or plaque number of all viruses tested that contained A_2 neuraminidase. In the chick embryo and in cell culture, low dilutions of antiserum reduced the yield of virus. True neutralization of virus in the chick embryo did not occur. We conclude that an antiserum specific for A_2 neuraminidase influenced the yield and release of virus from influenza virus-infected cells.

Recombination of A_0/NWS and A_2/RI/5$^+$ influenza viruses produced a virus, X-7, with A_0 hemagglutinin and A_2 neuraminidase (4, 7). When X-7 virus was backcrossed with A_2/RI/5$^+$, another hybrid was produced [X-7 (F1)] that was identical with X-7 in antigenic constitution, but which reacted differently in plaque-inhibition tests (3, 4). Virus X-7 (F1) and A_2 wild-type virus have twice the amount of A_2 enzyme as recombinant, X-7 (R. G. Webster, W. G. Laver, and E. D. Kilbourne, *in press*). The derivation of X-7 and X-7 (F1) viruses is diagrammed in Fig. 1. It is clear that viral genetic recombination has effected a segregation of hemagglutinin and enzyme proteins. Furthermore, Laver and Kilbourne (7) have demonstrated that A_0/NWS (and hence X-7) hemagglutinin is denatured during disruption of these viruses with sodium dodecyl sulfate (SDS) so that subsequent electrophoresis of X-7 viral proteins results in a separation of A_2 neuraminidase from residual contaminating hemagglutinin. This separation is not possible with the wild-type A_2 parent in which both hemagglutinin and neuraminidase resist denaturation and have the same electrophoretic mobility. Thus, with X-7 and X-7 (F1) viruses the separated enzyme can be eluted from the cellulose acetate strip as a presumably pure antigen uncontaminated with demonstrable biological or serological activity other than that associated with the neuraminidase (7). This paper describes the in vitro antiviral activity of influenza A_2-specific neuraminidase antiserum prepared by immunization with the isolated enzyme of X-7 (F1). A related paper discusses the effects in mice of active and passive immunization with the enzyme and its antiserum (J. S. Schulman, M. Khakpour, and E. D. Kilbourne, *to be published*).

MATERIALS AND METHODS

Cells. Clone 1-5C-4, derived from the Wong-Kilbourne variant of the human aneuploid Chang conjunctival cell line, was used for plaque-reduction tests (11).

Viruses. Most of the viruses employed have been described in earlier reports. These include A_2/RI/5$^+$, A_2/RI/5$^-$, A_0/NWS, and A_1/CAM, and recombinants X-1L, X-7(F1), X-9, and CAMr$^-$, (4, 12). Recombinant X-12 is an A_0A_2 hybrid derived from recombination of A_0/NWS and RI/5$^-$. A/Equi$_1$ was obtained through the courtesy of George Hirst. X-15 is a hybrid of A/Equi$_1$ and the A_2 recombinant X-1L (E. D. Kilbourne, Science, *in press*).

Plaque-assay technique. Methods for assay of plaques and for plaque inhibition with antisera have been published in detail (3).

Neutralization tests. Tests were carried out in

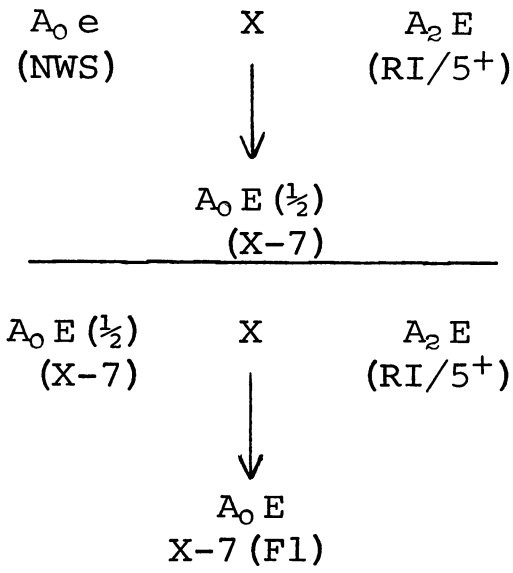

FIG. 1. *Derivation of recombinant X-7 (F1), source of A_2 neuraminidase (E) used in immunization for production of neuraminidase antiserum. A_0 and A_2 indicate nature of major (hemagglutinin) antigen; e and E indicate A_0 and A_2 enzymes, respectively. A_2 enzyme complement of X-7 is one-half that of A_2/RI/5 and X-7 (F1) viruses.*

groups of four 10- to 11-day chick embryos. Details are provided in Results.

Hemagglutination-inhibition tests. Hemagglutination-inhibition tests were performed by methods described previously (14).

Neuraminidase assay and enzyme-inhibition tests. Enzyme assays and enzyme-inhibition tests were performed with a fetuin substrate as described previously (7). Viruses employed in enzyme assays were grown in the allantoic sac of 10- to 11-day-old chick embryos and were stored as allantoic fluid suspensions at −95 C. In most instances, diluted allantoic fluid suspensions of freshly thawed intact viruses were used as enzyme, and they were adjusted so that sufficient *N*-acetyl neuraminic acid was liberated to give optical density (OD) readings of 0.300 to 0.900 at 549 mμ in a Bausch & Lomb Spectronic-20 colorimeter or in a 1.0-cm cell in a Beckman DU-2 spectrophotometer. Titers of antisera are expressed as that dilution which causes 50% inhibition of the neuraminidase activity of X-7 (F1) virus adjusted to give OD readings in the presence of dilutions of normal rabbit serum of 0.300 to 0.380.

Isolation of A_2 neuraminidase used in immunization. Virus X-7(F1), purified by adsorption-elution on red cells, differential centrifugation, and sedimentation through a sucrose gradient (7), was disrupted with SDS, and the protein components were separated by electrophoresis on cellulose acetate as described previously (6).

The protein band with A_2-type neuraminidase activity was eluted from the cellulose acetate strips with 0.15 M NaCl (7). (The enzyme from 12 strips was eluted with 7.0 ml.) The enzyme solution was placed in an ice bath overnight, and SDS which precipitated was removed by centrifugation. The enzyme in the supernatant fluid was freed further from SDS and buffer salts, and, at the same time, was concentrated by cold acetone precipitation. The solution of enzyme (7.0 ml) was cooled to 0 C, and cold (−20 C) acetone (25 ml) was added slowly with constant stirring. The mixture was allowed to stand overnight in an ice bath and then was centrifuged at 0 C (2,500 rev/min for 15 min). The supernatant liquid was poured off and discarded, and the tube drained for a few minutes at 0 C. The enzyme precipitate was then dissolved in cold 0.15 M NaCl (1.0 ml). Incubation of 1 μliter of this preparation with fetuin for 30 min in the standard enzyme assay gave an OD reading of 0.365.

RESULTS

Immunization procedure. Six male rabbits (1.3 to 1.8 kg) were injected in the footpad with 0.3 ml of a mixture of equal parts of aqueous dilutions of isolated neuraminidase and complete Freund's adjuvant. Antigen dilutions of 1:17, 1:67, and 1:267 induced neuraminidase antibody titers of 1:5,000 or more, 72 days after injection, but antigen diluted 1:1,069 or 1:4,275 did not induce neuraminidase-inhibiting antibody. In one rabbit (R-296) injected with the lowest dilution (1:10) of enzyme, serum specimens were obtained for study immediately prior to injection of antigen, 13, 22, 40, 47, 106, and 159 days afterward. A booster injection identical to the initial one was given on the 40th day. As presented in Table 1, antibody to the A_2 neuraminidase of X-7 (F1) virus appeared promptly in high titer, but hemagglutination-inhibiting antibody for X-7 and X-7 (F1) (hemagglutinin = A_0) appeared

TABLE 1. *Increase in serum anti-A_2 neuraminidase activity after immunization*

Day after initial injection	Log_{10} serum titer		
	Enzyme inhibition[a]	Hemagglutination inhibition[b]	Plaque-size reduction[c]
0	0.3	1.0	2.0
13	3.3	1.0	3.8
22	3.5	1.0	5.0
40	3.8	—[d]	—[d]
47[e]	4.0	1.3	6.6

[a] Dilution of antiserum producing 50% inhibition of enzyme activity of X-7(F1) virus diluted to give an optical density reading of 0.290 to 0.320.
[b] With X-7 and X-7(F1) viruses.
[c] With X-7 virus.
[d] Not tested.
[e] At 7 days after booster injection.

only in very low titer (1:20 dilution) after the booster injection. Plaque size-reducing (PSR) activity was demonstrable with high dilutions of serum (titer > 1:1,000,000 with the 47-day specimen). PSR has been correlated with inhibition of the viral enzyme (3). Not unexpectedly, the curves of direct enzyme inhibition and PSR were not parallel, as the former effect involved direct inactivation of enzyme in vitro, but PSR was an indirect measure of inhibition of viral replication (3). The enzyme-inhibition titration of the 47-day serum is shown in Fig. 2.

Evidence for the A_2 specificity of viral neuraminidase inhibition by the R-296 antiserum produced to the neuraminidase fraction of X-7 (F1) virus. Serum obtained 22 days after immunization with the neuraminidase fraction of X-7 (F1) virus was tested at a 1:100 initial dilution for its capacity to inhibit enzymatic (neuraminidase) activity of influenza A viruses of various subtypes. The homologous enzyme-inhibition titer of this serum was 1:3,000. The data from an experiment comparing the 0- and 22-day sera are given in Table 2. Significant inhibition of viral neuraminidase activity was found only with the wild-type A_2 strains RI/5$^+$ and RI/5$^-$ or with recombinants derived from them [X-1L, X-7, X-7 (F1), and X-15] in which inhibition of enzymatic activity with A_2 antiserum had previously been demonstrated. In this table, and in subsequent ones, the A_2 enzyme is represented as E, and the less stable enzymes of the A_0 and A_1 subtypes are represented as e and e′, respectively. Results obtained with the same viruses and the 47-day serum were the same as the data presented in Table 2. We concluded that the antiserum is specific for the A_2 neuramini-

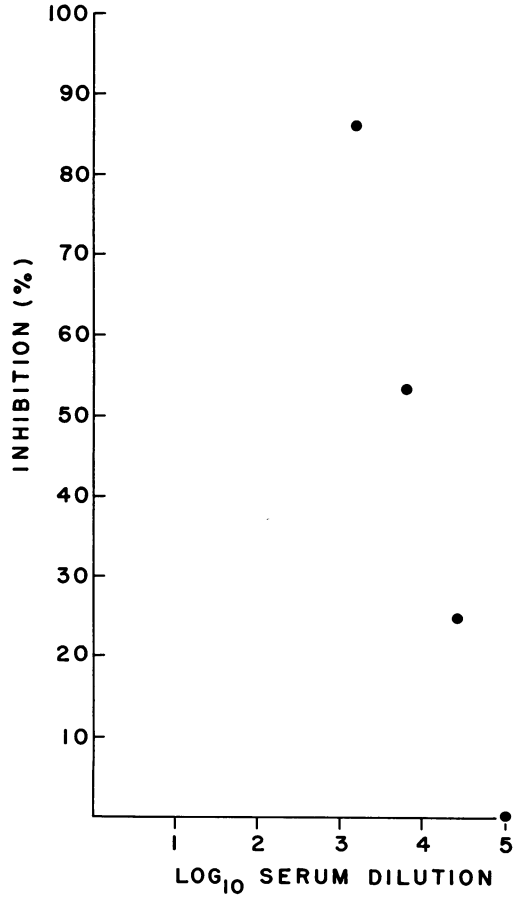

FIG. 2. *Titration of the R-296 47-day antiserum for neuraminidase antibody. Enzyme is X-7 (F1) virus suspension.*

TABLE 2. *Specificity for A_2 viral neuraminidase of R-296 neuraminidase antiserum*

Virus	Surface Antigens		Antiserum[a] (day)	OD[b]	Inhibition (%)
	Hemagglutinin	Enzyme			
RI/5$^+$	A_2	E(A_2)	0	.520	96
			22	.020	
RI/5$^-$	A_2	E(A_2)	0	.620	93
			22	.030	
X-1L	A_2	E(A_2)	0	.775	91
			22	.065	
X-7	A_0	E(A_2)	0	.560	94
			22	.025	
X-7(F1)[c]	A_0	E(A_2)	0	.305	90
			22	.020	
X-12	A_0	E(A_2)	0	.690	93
			22	.045	
Equi$_1$	Equi	(equi)	0	.415	12
			22	.365	
X-15	Equi	E(A_2)	0	.875	89
			22	.095	
CAM	A_1	e′(A1)	0	.415	0
			22	.455	
CAMr$^-$	A_1	e′(A1)	0	.600	27
			22	.440	
NWS[d]	A_0	e(A_0)	[e]	.130	0
			22	.150	
X-9[d]	A_2	e(A_0)	[e]	.150	0
			22	.170	

[a] Serum specimens from rabbit R-296 obtained prior to (0 day) and 22 days after injection of neuraminidase in Freund's adjuvant. Serum diluted 10^{-2} = 30 times the concentration required for 50% inhibition of neuraminidase activity of X-7(F1) virus.

[b] Optical density reading at 549 mμ.

[c] Source of enzyme used in immunization.

[d] At 18-hr incubation of virus-antiserum mixture with substrate.

[e] Another normal rabbit serum used as control.

dase. The suggestion of inhibition of CAMr$^-$ (27%) was not confirmed in additional experiments in which virus was tested with a range of antiserum dilutions from 1:20 to 1:1,280. Furthermore, neither CAM nor CAMr$^-$ antisera were inhibitory to the activity of the isolated X-7 (F1) enzyme.

Hemagglutination-inhibition activity of R-296 neuraminidase antiserum. Antiserum to the neuraminidase (Table 1) was virtually devoid of hemagglutination-inhibition activity with the virus from which its antigen was derived, X-7 (F1) [A_0 E]. The antiserum did not inhibit hemagglutination with the wild-type A_0 strain (NWS) or with a recombinant X-9 (4) that contained A_2 hemagglutinin but lacked A_2 neuraminidase (Table 3). Thus, the low-titer hemagglutination-inhibiting activity of the antiserum with X-7 and X-7 (F1) viruses is readily explained as a consequence of reaction with their A_2 enzyme components, and it does not seem to be effected by antibodies to a cryptic A_0 or A_2 hemagglutinin component contaminating the extracted neuraminidase used in immunization.

We noted that the R-296 47-day serum had appreciable hemagglutination-inhibiting activity with the inhibitor-sensitive, antibody-avid I (5) or "+" (1) A_2E strains RI/5$^+$ and X-1L (Table 3). But the antiserum did not inhibit the inhibitor- and antibody-resistant (i, "−") RI/5$^-$ strain that is antigenically identical to RI/5$^+$ (1). Thus, this reactivity may also be explained by the reaction at the enzyme site and does not necessarily suggest the presence in R-296 antiserum of influenza virus antibodies other than antineuraminidase. Indeed, absorption of antiserum with 5.1×10^4 or 6.4×10^2 hemagglutinating units of concentrated A_0/NWS or A_2/X-9 viruses, respectively, neither reduced the HI titer for A_2/RI/5$^+$ or A_2/X-1L nor for the Equi-A_2 recombinant, X-15, which was markedly inhibited in HI tests with R-296 antiserum (Table 3). It has not been possible to demonstrate in X-15 virus any A_2 component other than neuraminidase (e.g., there is no cross-reactivity with X-9 (A_2e) virus). The unusual properties of X-15 are the subject of another report (E. D. Kilbourne, Science, *in press*). The low-level HI reaction with the A_1 viruses CAM and CAMr$^-$ was unexpected and puzzling. Neither virus showed evidence of possessing A_2-like neuraminidase nor reacted in enzyme-inhibition tests with R-296 antiserum (Table 2). However, HI crosses of these viruses with A_0/NWS (9) and with A_2/RI/5$^+$ and X-1L are demonstrable, which suggests the presence of antigens in common—perhaps a hemagglutinin of CAM and the A_2 neuraminidase.

Effect of neuraminidase antiserum on plaque formation. Monolayers of clone 1-5C-4 cells in 60-mm petri dishes were inoculated with 50 to 100 plaque-forming units (PFU) of the influenza viruses listed in Table 4. Agar overlays containing serial fourfold dilutions of R-296 antiserum (47-day bleeding) were then added, and these dishes were examined 4 days later after crystal violet staining for reduction in plaque number (plaque inhibition, PI) or plaque size PSR (3). As noted in Table 4, extremely high dilutions of antiserum (>1:1,000,000) affected plaque formation by all viruses that contained A_2 neuraminidase (E), but

TABLE 3. *Hemagglutination-inhibiting activity of specific A_2 neuraminidase antiserum*[a]

Virus	Surface antigens		Hemagglutination-inhibition titer[b]
	Hemagglutinin	Enzyme	
X-7	A_0	E(A_2)	1.3
X-7(F1)[c]	A_0	E(A_2)	1.3
NWS	A_0	e(A_0)	<1.0
X-9	A_2	e(A_0)	<1.0
RI/5$^+$	A_2	E(A_2)	1.9
RI/5$^-$	A_2	E(A_2)	<1.0
X-1L	A_2	E(A_2)	2.8
Equi$_1$	Equi	(equi)	<1.0
X-15	Equi	E(A_2)	3.1
CAM	A_1	e′(A_1)	1.6
CAMr$^-$	A_1	e′(A_1)	1.6

[a] Rabbit R-296 (47-day bleeding).

[b] Reciprocal of serum dilution at end point ($\log_2$).

[c] Virus from which immunizing enzyme antigen was derived.

TABLE 4. *Effect of antineuraminidase [anti-E (A_2)] on plaque formation*[a]

Virus	Antigens		Log_{10} plaque-reducing titer[b]	
	HA	Enzyme	PSR	PI
X-7	A_0	E(A_2)	6.6	—
X-7(F1)	A_0	E(A_2)	—	6.6
X-12	A_0	E(A_2)	6.6	—
X-1L	A_2	E(A_2)	—	6.6
NWS	A_0	e(A_0)	<2.7	<2.7
X-9	A_2	e(A_0)	<2.7	<2.7
CAM	A_1	e′(A_1)	<2.7	<2.7
CAMr$^-$	A_1	e′(A_1)	<2.7	<2.7
NWSr$^-$	A_0	e′(A_1)	<2.7	<2.7

[a] PSR = reduction in plaque size without reduction in plaque number. PI = reduction in plaque number (plaque inhibition).

[b] Final dilution of antiserum incorporated in agar overlay effective in plaque reduction (50% PI or PSR).

plaque formation of other A_0, A_1, and A_2 viruses was not influenced at the lowest dilution used (1:500). The results with X-7, X-7 (F1), and X-1L viruses were consistent with the patterns reported previously for these viruses when titrated against $A_2/RI/5^+$ antiserum, i.e., complete plaque inhibition of X-1L and of X-7 (F1) which contained more neuraminidase (Webster et al., *to be published*) and PSR of X-7 (2). Virus X-12 was apparently identical antigenically with X-7, but it had derived its neuraminidase from $RI/5^-$ rather than from $RI/5^+$.

Neutralization in chick embryos. Several viruses containing differing hemagglutinin moieties and either E (A_2) or e (A_0) enzyme components were serially diluted in decimal series. To each virus dilution, a 1:10 dilution of either the 0-day or 22-day serum of R-296 was added, and the mixtures were maintained at 4 C for 1 hr before intra-allantoic inoculation of 10-day-old chick embryos. The allantoic fluids were harvested after the eggs were incubated for 40 hr and tested at dilutions of 1:4 with 0.5% suspensions of human group O RBC for hemagglutinin, as in a conventional neutralization titration. Titration end points were read on the basis of this result (Table 5). We noted that titration end points of viruses containing the A_2 enzyme (E) were reduced 1 to 3 logs by the 22-day antineuraminidase serum. However, when allantoic fluids from eggs negative for hemagglutinating virus were inoculated at a 1:10 dilution into other eggs, in an attempt to demonstrate infective virus below the threshold of detectable hemagglutination, the significant difference in titration end points disappeared. That is, some eggs inoculated with high dilutions of virus and neuraminidase antiserum contained infective virus, although in the conventional test they had been read as "negative." Furthermore, hemagglutination titrations of positive allantoic fluids from X-7 (F1) virus infection demonstrated lower yields of hemagglutinating virus in eggs infected in the presence of neuraminidase antiserum (Table 6). Thus, true neutralization—i.e., reduction in initial infective dose—was not effected with the neuraminidase antiserum in eggs, but rather a reduction in viral yields resulted after inoculation. This effect is analogous to PSR and to the results obtained with X-7 virus in chorioallantoic membrane fragments in vitro with antineuraminidase antibodies obtained by viral absorption from X-7 antiserum (13).

Preinoculation neutralization of plaque formation. Mixtures of 100 to 150 PFU of virus (X-7, X-7 (F1), X-1L, NWS, and X-9) and serial twofold dilutions of 22-day R-296 antiserum were added to clone 1-5C-4 cell monolayers. The 0-day serum of R-296 served as a control. After a 30-min absorption period, monolayers were washed once with 10 ml of 199, and agar overlays were added as usual. Plaques were measured and counted after crystal violet staining, following 4 days of incubation of the dishes. Titration end points with the various viruses were as follows: X-7, 1:24; X-7 (F1), 1:192; X-1L, 1:384; NWS and X-9, <1:8. This apparent neutralizing reactivity against E-containing viruses in the cell culture system was low level (i.e., $<10^3$), compared to the high activity of antiviral antisera in similar titrations (10^4 to 10^5); this suggested that the large number of antineuraminidase antibody

TABLE 5. *Reduction in viral yields by anti-A_2 neuraminidase in chick embryos without true neutralization*

Expt	Virus	Antigens		Titration[a] of virus in eggs with anti-E or normal serum[b]				Titer difference (NRS-Anti-E)	
				1st Passage		2nd Passage			
		HA	Enzyme	NRS	Anti-E	NRS	Anti-E	Passage 1	Passage 2
1	X-7 (F1)	A_0	E	7.0[c]	4.0	7.0	7.0	+3.0	0
2	X-7 (F1)	A_0	E	6.3	4.0	6.3	5.5	+2.3	+0.8
2	X-1L	A_2	E	8.0	5.3	8.5	7.8	+2.7	+0.7
2	X-15	Equi	E	7.8	6.8	7.8	8.4	+1.0	−0.6
3	NWS	A_0	e	7.7	7.8	—	—	0.0	—
3	X-9	A_2	e	7.5	7.3	—	—	+0.2	—

[a] Each of a decimal series of virus dilutions was added to a 1:10 dilution of either R-296 22 day or normal serum, maintained for 60 min at 4 C and was then inoculated into chick embryos; allantoic fluids were harvested after 40 hr of inoculation at 35 C and were tested at 1:4 dilution for hemagglutinating virus. Titration end points were read on the basis of this result and again after subinoculation into chick embryos of fluids negative for hemagglutinin (2nd passage).

[b] Anti-E = neuraminidase antiserum (R-296, 22-day bleeding); NRS = normal rabbit serum.

[c] Log_{10} EID_{50} titer.

molecules demonstrated in enzyme inhibition and PSR titrations (Table 1) is relatively inefficient in preinoculation neutralization of the virus. Cell cultures were not titrated for residual unneutralized virus as was done in the chick-embryo system; thus, comparison of results with that system was not possible.

Effect of neuraminidase antiserum on yield and release of X-7 (A_0E) virus from clone 1-5C-4 cells. Evidence was obtained from the following experiment that the essential effect of antineuraminidase antibody in cell cultures is on yield of virus from cells. Clone 1-5C-4 monolayers were inoculated with either 5.0 or 0.05 PFU per cell of X-7 virus. After the cells were washed, maintenance medium, containing 1% of R-296 22-day or 0-day serum, was added to the cultures (Table 7), which were then incubated for 20 hr—a period optimal for maximal production of infective virus following high multiplicity of infection (11). Culture fluids were removed and centrifuged at 8,000 × *g* for 5 min. Supernatant fluids were diluted in a 10-fold series and inoculated into eggs for determination of EID_{50} yields of infective virus. At the lowest dilution of culture fluid employed (10^{-1}), it can be calculated that the final dilution of the original serum was at least 10^{-3} in the chick-embryo allantoic sac (0.1 ml of 10^{-1} inoculated into 10 ml of allantoic fluid). Thus, the dilution was less than the amount required to produce reduction of virus yields in the chick embryo or to affect testing of allantoic fluid harvests for hemagglutinin. Reduction (10- to 50-fold) in the yield of fluid-phase infective virus was demonstrated in this experiment (Table 7).

TABLE 6. *Reduction in yield of hemagglutinating X-7(F1) virus after inoculation of eggs with mixtures of virus and A_2 neuraminidase antiserum*[a]

Virus dose, $\log_{10}$ (EID_{50})	Egg no.	Hemagglutination titer	
		NRS[b]	Anti-E[c]
3.3	1	1,024[d]	32
	2	256	64
	3	512	8
	4	512	<4
2.3	1	512	16
	2	256	<4
	3	1,024	<4
	4	256	32

[a] Experiment conducted as summarized in footnote to Table 5.
[b] Normal rabbit serum (R-296, 0 day).
[c] Neuraminidase antiserum (R-296, 22 day).
[d] Reciprocal of virus (allantoic fluid) dilution at the end point.

TABLE 7. *Reduction in yield of virus X-7 from clone 1-5C-4 cells in the presence of A_2 neuraminidase antiserum*

Virus/cell input multiplicity (PFU)	Serum in medium	$\log_{10}$ EID_{50} yield/10 ml[a]
5	Anti-E[b]	4.0
5	NRS[c]	5.7
0.05	Anti-E	<3.0[d]
0.05	NRS[c]	4.0

[a] Total volume of medium per petri dish was 10 ml.
[b] Anti-neuraminidase antiserum (R-296, 22-day bleeding, 1:100 dilution).
[c] Normal rabbit serum (R-296, 0-day bleeding, 1:100 dilution).
[d] For this determination, 0.1 ml of medium was tested at 10^{-1} dilution.

DISCUSSION

Genetic recombination has effected the segregation of influenza A_2 viral neuraminidase from other A_2 viral proteins with the production of an A_0A_2 hybrid [X-7 (F1)] in which the A_0 viral proteins are fortuitously vulnerable to denaturation with SDS—the detergent used for disruption of influenza viruses for study of their viral proteins. Consequently, A_2 neuraminidase has been isolated in apparently pure form, after electrophoresis, and has been employed as an antigen for the production of specific neuraminidase antibody.

This paper is concerned with a detailed analysis of the antiviral activity of antiserum produced in response to immunization with the isolated neuraminidase. A unique feature of the present system is the availability for use in serological reactions of recombinant viruses in which the hemagglutinin and enzyme moieties of the A_0 and A_2 subtypes are carried in different combinations. Thus, the reactivity of the neuraminidase antiserum with certain A_2 viruses in HI tests is almost certainly not an indication of contamination of the enzyme antigen with A_2 hemagglutinin protein because similar reactivity is not detectable with an A_2 recombinant lacking the A_2 enzyme. Rather, it can be inferred that neuraminidase antibody can cause hemagglutination-inhibition of certain antibody-avid viruses (A_2/RI/5$^+$ and X-1L) containing the homologous enzyme. Similarly, the absence of contaminating A_0 hemagglutinin in the immunizing preparation is shown by the complete nonreactivity of A_2 neuraminidase antiserum in all serological tests of A_0 viruses lacking the A_2 neuraminidase.

A summary of the activity of specific A_2 neuraminidase antiserum in various serological tests appears in Table 8. It is clear that, regardless of the nature of its hemagglutinin, reactivity of a

TABLE 8. *Summary of effects of antiserum specific for influenza A_2 neuraminidase*

Virus	Log_{10} titers			
	Hemagglutination inhibition	In ovo neutralization	Enzyme inhibition	Plaque reduction[a]
A_0e	<1.0	<1.0	<2.0	<1.7
A_2E	<1.0[b]	<1.0[c]	4.0	6.6
A_0E	1.3	<1.0[c]	4.0	6.6
A_2e	<1.0	<1.0	<2.0	<1.7
A_1e'	1.6[d]	<1.0	<2.0	<1.7
Eq eq	<1.0	<1.0	<2.0	[e]
Eq E	3.1	<1.0†	4.0	[e]

[a] PI or PSR.

[b] Reactive with the "I" or "+" strains $RI/5^+$ and X-1L, only.

[c] Reduced viral yield in first passage, but no true neutralization.

[d] Reactive with highest titer (47 day and 106 day) sera only.

[e] Virus does not produce plaques.

virus in enzyme inhibition or in plaque-reduction tests is dependent upon its possession of neuraminidase that is identifiable as A_2 in tests with A_2 viral antiserum. It is significant that the high-titer antiserum to A_2 neuraminidase, prepared in this study by hyperimmunization with antigen in adjuvant, is completely nonreactive with A_0e (NWS) virus that contains the envelope protein(s) (other than the neuraminidase) of the X-7 (F1) virus from which the enzyme was extracted. As measured in the plaque-reduction system, the titer of the antiserum with A_0E virus is 1:5,000,000, but no activity was detectable with A_0e with a 10,000-fold lesser dilution (i.e., 1:500). The antiserum-in-overlay technique with PSR as an end point appears to be an extremely sensitive method for the detection and identification of viral neuraminidase as antigen; thus, the lack of effect of the R-296 antiserum on any but the A_2 neuraminidase-containing viruses is reassuring with respect to the specificity of the antiserum and is concordant with the results obtained in enzyme-inhibition tests.

In the chick-embryo neutralization test in which antibody remains present through the viral growth cycle, true neutralization does not occur, but only reduction in viral yield after infection occurs, as shown by the demonstration of infective virus in eggs negative for hemagglutinin. This reduction in viral yield in chick embryos effected by antibody to the viral enzyme is consistent with the depressed yield of virus from cell cultures described in this present study. The results are also consistent with the phenomenon of reduction in plaque size but not in plaque number (PSR), occasioned by the continued presence of antibody to the viral enzyme in agar overlays in cell cultures (3). A similar effect was noted with fowl plague virus in chick-embryo tissue cultures, i.e., non-neutralization of infectivity but reduction of viral plaque size, as well as reduction in cell-associated hemagglutinin and enzyme (10). In chick-chorioallantoic membrane cultures in liquid media, the continued presence of enzyme antibody is necessary for apparent neutralization of virus (13). All these studies in different laboratories using different systems indicate that the primary role of viral neuraminidase is not in the initial steps of infection but rather in later events related to the release and spread of virus from infected cells.

The antigenic dissimilarity of the neuraminidase and hemagglutinin of influenza virus, suggested previously by Rafelson's studies (9), has been clearly demonstrated by our experiments, which also support the earlier inference that the enzyme and hemagglutinin sites on the influenza virion are not identical (2, 8).

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