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Protective Effects of Specific Immunity to Viral Neuraminidase on Influenza Virus Infection of Mice

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Antibody specific for viral neuraminidase can be demonstrated in mice following (i) pulmonary infection with influenza virus, (ii) immunization with ultraviolet-inactivated influenza virus, (iii) immunization with isolated neuraminidase of influenza A_2 virus, and (iv) passive immunization with sera of rabbits immunized with isolated A_2 neuraminidase. Neuraminidase antibody produced by any of these methods exerts a profound inhibiting effect on virus replication in the lungs of mice challenged with strains of virus having homologous neuraminidase protein, even in the absence of hemagglutinating inhibiting antibody to the challenge virus, and results in markedly decreased pulmonary virus titers and diminished lung lesions. These observations suggest that antineuraminidase immunity may play a significant role in the protection against influenza virus challenge observed in mice after infection or artificial immunization.

It is now well established that hemagglutinin and neuraminidase are antigenically distinct proteins of the envelope of influenza virus, and that by genetic recombination hybrid (recombinant) viruses can be produced in which hemagglutinin is derived from one parental virus and neuraminidase from the other (7, 8, 11). Such antigenically hybrid viruses in which hemagglutinin and neuraminidase proteins from different subtypes have been segregated have proved useful in the isolation of neuraminidase free of demonstrable hemagglutinin protein (11) and in the production of specific antibody to viral neuraminidase (7).

In aneuploid cell culture, in ovo, and in chick shell-membrane systems, neuraminidase antibody is non-neutralizing except in high concentrations, but it partially inhibits virus replication by its effect on the release and yield of influenza virus from cells (7, 14, 16; R. G. Webster, W. G. Laver, and E. D. Kilbourne, *submitted for publication*).

In earlier studies of mice immunized by infection with antigenically hybrid influenza A viruses, equivalent protection was found after challenge with viruses which contained either the same hemagglutinin or the same "minor" (neuraminidase) antigen possessed by the immunizing virus (9).

We now report a further comparison of the relative effectiveness of specific immunity to viral neuraminidase and immunity to viral hemagglutinin in protecting mice against challenge with influenza virus infection. In these experiments, antibody to neuraminidase was produced in mice (i) by infection with recombinant viruses in which hemagglutinin and neuraminidase were derived from parents of different subtypes, (ii) by injection of ultraviolet-inactivated preparations of the same viruses, (iii) by injection of purified A_2 neuraminidase, or (iv) by passive immunization with rabbit antibody to the enzyme.

MATERIALS AND METHODS

Cells and plaque assay. Clone 1-5C-4, derived from the Wong-Kilbourne variant of the human aneuploid Chang conjunctival cell line, was used for plaque-reduction tests (15). The methods for assay of plaques and for plaque inhibition with antisera have been published in detail (5, 15).

Viruses. Most of the viruses employed have been described in earlier reports (9). These include A_0/NWS and A_2/Jap. 305 (mouse adapted) and the following recombinants: unadapted and mouse-adapted strains of X-7, X-3, X-1L, and X-9 (17 passages in mice), X-7(F1), and X-15 (hybrid of A/equine 1 containing A_2 neuraminidase) (6). The antigenic designation of these viruses (Table 1) was made in accordance with a system described previously (7, 8).

Ultraviolet inactivation of virus. In some instances, allantoic fluid seed viruses were inactivated by ultra-

violet (UV) irradiation prior to parenteral inoculation into mice. Fluids were centrifuged at 8,000 rev/min, dialyzed at *p*H 7.2 overnight, and then subjected to UV light from a 7.5-w GE lamp at a distance of 17.8 cm for 2 min. After this procedure, residual infectivity for eggs could be demonstrated only with undiluted fluids in the presence of cortisone. The fluids then were adjusted to identical hemagglutination titers prior to inoculation of mice.

Mice. Specific pathogen-free male Manor Farm mice (MF-1) 8 to 16 weeks of age were used.

Eggs. White Leghorn chick embryos 10 to 12 days of age were used to assess the infectivity titer of all tissues.

Hemagglutination-inhibition tests. Hemagglutination tests with mouse and rabbit sera were performed by methods described previously (17).

Enzyme-inhibition tests. Enzyme-inhibition tests with mouse and rabbit sera were carried out with a fetuin substrate according to methods described previously (7, 11). Measurements of inhibition of A_2 neuraminidase (E) were made with X-7(F1) virus adjusted to give optical density readings of 300 to 800 at 549 nm in a Bausch and Lomb Spectronic-20 colorimeter or Beckman DU-2 spectophotometer. Measurements of inhibition of A_0 neuraminidase (e) were made with the same substrate using an 18-hr incubation period of X-9 (A_2e) virus and substrate. Titers of antisera are expressed as the dilution at which 50% inhibition of neuraminidase activity occurred.

Isolation of A_2 neuraminidase. The purification and disruption of X-7(F1) virus and the isolation and elution of the neuraminidase protein from cellulose acetate strips were carried out by methods described previously (11).

Virus titrations. Lungs were removed at designated periods after infection and ground in glass tubes according to techniques described previously (12). Serial 10-fold dilutions of ground lung suspensions were inoculated into eggs, and after 40 hr of incubation allantoic fluids were harvested and were tested for hemagglutination with human "O" red cells at a 1:4 dilution. Titration end points were calculated in terms of the dilution of lung tissue infecting 50% of the chick embryos.

Scoring of pulmonary lesions. A modification of the maximal score method was used, in which the extent of pulmonary lesions was expressed as a percentage of the total lung surface (4).

Aerosol infection of mice. The apparatus and procedures used to generate aerosols of infectious virus have been described previously (12). During the periods in which they were inside the aerosol chamber, mice were exposed to an estimated 10 to 100 mouse infective doses of the challenge viruses employed.

Intranasal inoculation. In some experiments, rabbit antiserum was delivered intranasally to mice. Mice were lightly anesthetized with ether, and three drops (0.05 ml) of the appropriate serum was delivered into the nostrils through a 26-gauge needle.

Bronchial washings. Mice were killed by cervical fracture, and the trachea was dissected free of the esophagus and surrounding connective tissue. A no. 20 adapter was inserted into the trachea through a small hole and tied in place by thread. An amount of 1 ml of sterile saline (0.1% gelatin) was injected into the trachea and lungs and then was aspirated back into a sterile syringe (0.4 to 0.8 ml was recovered). These fluids were treated as undiluted bronchial washings, and all subsequent dilutions were expressed accordingly.

TABLE 1. *Antigenic designation of viruses employed in the present study*

Virus	Hemagglutinin subtype	Neuraminidase subtype	Antigenic designation
X-7.....	A_0	A_2	A_0E[a]
X-7(F1)...	A_0	A_2	A_0E
X-3.....	A_0	A_0	A_0e[b]
X-1L.....	A_2	A_2	A_2E
X-9.....	A_2	A_0	A_2e
X-15.....	A/equine 1/56	A_2	Eq E
NWS.....	A_0	A_0	A_0e
Jap. 305...	A_2	A_2	A_2E

[a] E = A_2 neuraminidase.
[b] e = A_0 neuraminidase.

RESULTS

Demonstration of neuraminidase antibody in mice after influenza virus infection. Mice were infected with 100 mouse infective doses of A_0/NWS or A_2/Jap. 305 virus or were exposed to an aerosol of saline. Serum specimens and bronchial washings from 10 mice in each group were separately pooled 4 weeks later for antibody determination. Hemagglutinating-inhibiting (HI) activity was measured against the two infecting viruses and against recombinant X-15 (equine E) virus [a virus inhibitable in hemagglutination-inhibition tests with antibody to A_2 neuraminidase (E) (6)]. Enzyme-inhibiting antibody was measured against X-7 (F1) (A_0E) and X-9 (A_2e) recombinant viruses, and plaque size-reducing activity was titrated in human conjunctival cells infected with X-9 (A_2e) or X-7 (A_0E) viruses under antibody in agar overlays. The results (Table 2) demonstrate that, following influenza virus infection of mice, hemagglutinating-inhibiting antibody appeared in bronchial secretions, as well as in the serum, and that antibody to the neuraminidase component of the infecting virus was present in the serum. This neuraminidase antibody was demonstrated by inhibition of enzymatic activity of intact X-9 (A_2e) or X-7 (F1) (A_0E) virus and by plaque size reduction of viruses containing neuraminidase homologous to the neuraminidase antibody. The HI activity against X-15 virus in sera of mice previously infected with A_2/Jap. 305 virus is additional evidence of A_2

TABLE 2. *Titers of HI, enzyme-inhibiting (EI), and plaque size-reducing (PSR) antibody in sera and bronchial wash fluids obtained from mice 4 weeks after immunizing infection with A_0/NWS and A_2/Jap. 305 viruses*

Test	Virus		Virus infection			
			NWS		Jap. 305	
			Serum[a]	Bronchial wash[a]	Serum	Bronchial wash[a]
HI.....	NWS	A_0e	512[b]	16	<8	<8
	Jap. 305	A_2E	<8	<8	512	64
	X-15	EqE	<8	<8	128	<8
EI.....	X-9	A_2e	50	<2	<2	<2
	X-7(F1)	A_0E	<2	<2	2	<2
PSR....	X-9	A_2e	400	NT[c]	NT	NT
	X-7	A_0E	NT	NT	200	NT

[a] Pooled sera and bronchial wash fluid, 10 animals in each group.

[b] Reciprocal of dilution producing HI of 4 units of hemagglutinin; 50% reduction in enzymatic activity and reduction in plaque size.

[c] Not tested.

neuraminidase antibody, since the only nonequine viruses capable of inducing the formation of such antbody are those which contain A_2 neuraminidase (6). The absence of demonstrable enzyme-inhibiting antibody in bronchial wash fluids may well be the consequence of the large dilution factor involved in obtaining these fluids.

Immunization and reciprocal challenge of mice with hybrid viruses in which hemagglutinin and neuraminidase proteins have been derived from viruses of different subtypes. Mice were infected by exposure to an aerosol of 100 MID_{50} (mouse infectious doses) of mouse-adapted X-3 (A_0e), X-7 (A_0E), X-1L (A_2E), or X-9 (A_2e) viruses; control mice were exposed to a saline aerosol. Serum specimens from five animals were pooled for titration of HI and enzyme-inhibiting activity 4 weeks later, and the remaining mice in each group were challenged with each of the four recombinant viruses and with wild-type parental NWS (A_0e) and Jap. 305 (A_2E) viruses. Pulmonary virus titers were measured 48 hr after challenge. The results (Tables 3 and 4) demonstrate that 4 weeks after the immunizing infection mouse sera contained HI antibody against viruses that possessed the same hemagglutinin antigen as the immunizing virus and the enzyme-inhibiting antibody to the neuraminidase of the immunizing virus. Thus, following infection with X-7 (A_0E) virus which contains hemagglutinin protein derived from its A_0 parent and neuraminidase derived from its A_2 parent, mice had serum antibody to the A_0 hemagglutinin and to A_2 neuraminidase (E). Conversely, X-9 (A_2e) immunized mice had HI antibody to A_2 hemagglutinin and to A_0 neuraminidase (e). Following subsequent challenge with a virus containing the neuraminidase protein of the immunizing virus, but a different hemagglutinin, replication of the challenge virus in the lungs was inhibited by 99.9% or more. In some instances, this protection was so effective that no virus was demonstrable in the lungs 48 hr after challenge. When immunizing and challenge viruses contained the same hemagglutinin but different neuraminidase proteins, inhibition of replication of the challenge virus was greater. However, because of the logarithmic expression of titers, the increment of protection represents less than 0.1% of the virus titers in lungs of unimmunized animals. Interpretation of these results is complicated by evidence of heterotypic protection (13) expressed by low-level cross-reactivity in hemagglutination-inhibition tests and by lower pulmonary virus titers in instances where the immunizing and challenge viruses differed in their content of both neuraminidase and hemagglutinin proteins. It became important, therefore, to examine the effect of anti-neuraminidase immunity under experimental conditions where heterotypic immunity could not be demonstrated.

Challenge of mice immunized by infection with X-3 (A_0e), X-1L (A_2E), and X-9 (A_2e) recombinant viruses with X-15 (Eq E) virus. In preliminary studies in this laboratory with mice immunized by infection with influenza A_0 and A_2 viruses, no

TABLE 3. *Titers of hemagglutinating-inhibiting (HI) and enzyme-inhibiting (EI) antibody in sera of mice immunized by infection with A_0e, A_0E, A_2E, and A_2e recombinant viruses*

Determination	Immunizing infection[a]				
	X-3 A_0e	X-7 A_0E	X-1L A_2E	X-9 A_2e	Saline
HI titer vs.					
NWS (A_0e).....	512[b]	256	<16	<16	<16
Jap. 305(A_2E).....	<16	<16	64	64	<16
X-3 (A_0e).....	*256*	128	<16	32	<16
X-7 (A_0E).....	256	*256*	<16	<16	<16
X-1L (A_2E).....	<16	<16	*64*	64	<16
X-9 (A_2e).....	<16	<16	16	*128*	<16
X-15 (EqE).....	<16	64	128	<16	<16
EI titer vs.					
X-7(F1) virus(A_0E)..	2	50	50	<2	<2
X-9 virus (A_2e).....	40	<2	2	50	<2

[a] Four weeks before bleeding.

[b] Titers expressed as reciprocal of serum dilution producing HI with 4 units of hemagglutinin and 50% reduction of enzymatic activity.

TABLE 4. *Pulmonary virus titers of mice immunized by infection with A_0e, A_0E, A_2E, A_2e recombinant viruses challenged with the same hybrid viruses and with A_0/NWS and A_2/Jap. 305 influenza viruses*

Immunizing infection[a]	Challenge infection					
	NWS A_0e	Jap. 305 A_2E	X-3 A_0e	X-7 A_0E	X-1L A_2E	X-9 A_2e
X-3 (A_0e)	<1.7 (0/5)[b]	5.9 (5/5)	*<1.7 (0/5)*	<2.4 (2/5)	6.4 (5/5)	<3.7 (3/5)
X-7 (A_0E)	<1.7 (0/5)	3.6 (5/5)	<1.7 (0/5)	*<1.7 (0/5)*	<3.4 (3/5)	6.5 (5/5)
X-1L (A_2E)	6.2 (5/5)	<1.8 (1/5)	7.3 (5/5)	4.5 (5/5)	*<3.7 (3/5)*	<2.0 (1/5)
X-9 (A_2e)	<1.7 (0/5)	<1.7 (0/5)	<3.0 (2/5)	7.0 (5/5)	<1.7 (0.5)	*<1.7 (0/5)*
Saline	7.0 (5/5)	7.1 (5/5)	7.6 (5/5)	7.8 (5/5)	6.9 (5/5)	7.2 (5/5)

[a] By aerosol exposure 4 weeks prior to challenge.

[b] Mean titer EID_{50} $\log_{10}$ (numbers in parentheses = number with titer of 10^2 or greater per number exposed).

heterotypic protection was observed after challenge with A/equine$_1$/56 virus. Mice immunized by aerosol infection with nonadapted recombinant viruses X-3 (A_0e), X-7 (A_0E), X-1L (A_2E), and X-9 (A_2e) were challenged 4 weeks later with X-15 (Eq E) virus. Pulmonary virus titers of X-15 virus, 3 days after challenge, were appreciably reduced in those animals that had been immunized by infection with X-7 (A_0E) or X-1L (A_2E) viruses, both of which contain A_2 neuraminidase (E) identical to the enzyme component of the challenge virus (Table 5). In contrast, immunization by infection with X-3 and X-9 viruses, both of which lack the A_2 enzyme, had no effect on pulmonary virus titers 3 days after challenge with X-15 virus. Thus, in a situation in which heterotypic immunity related to hemagglutinin is not demonstrable, the immunizing effect of anti-neuraminidase immunity is apparent.

Immunization of mice with UV-inactivated recombinant viruses. Groups of mice were injected intraperitoneally with 0.5 ml of UV-inactivated X-7 (A_0E) or X-3 (A_0e) virus or saline at weekly intervals for 3 weeks. One week after the last injection, five mice from each group were bled, and the remaining mice were challenged by exposure to an aerosol of Jap. 305 (A_2E) virus. Pulmonary virus titers, lung lesions, and serum HI antibody titers were measured at intervals after challenge. The results (Table 6) demonstrate that immunization of mice with inactivated X-7 virus resulted in lower pulmonary virus titers and less extensive lung lesions following challenge with Jap. 305 (A_2E) virus. In contrast, only minimal evidence of heterotypic protection was observed as a result of immunization with X-3 (A_0e) virus. Thus, immunization with an inactivated recombinant virus (X-7) containing the same (A_2) neuraminidase protein as the challenge virus (Jap. 305) but a different (A_0) hemagglutinin

TABLE 5. *Effect of prior infection of mice with A_0e, A_0E, A_2E, and A_2e recombinant viruses on pulmonary virus titers 72 hr after challenge with recombinant X-15 (Eq E) virus*

Initial infection	X-15 (Eq E) challenge titer[a]
X-3 (A_0e)	6.6
X-7 (A_0E)	5.5
X-1L (A_2E)	4.2
X-9 (A_2e)	6.2
Saline	6.5

[a] EID_{50}, $\log_{10}$, five animals in each group.

resulted in appreciable protection against the challenge infection. In addition, it should also be noted that X-7 immunized mice demonstrated a more rapid serum HI antibody response to the challenge virus than saline or X-3 immunized mice. Thus, 6 days after challenge when HI antibody at a titer of 1:8 or greater was not demonstrable in any of the sera of mice from either of the other groups, five of the five X-7 immunired animals had HI antibody to Jap. 305 virus in their sera.

Immunization of mice with isolated A_2[X-7(F1)] neuraminidase. Mice were injected subcutaneously with 0.3 ml of a mixture of equal parts of saline and complete Freund's adjuvant or of a 1:16 dilution of isolated A_2 enzyme (E) derived from X-7(F1) (A_0E) and adjuvant. The injections were repeated 40 days later, and 7 days after the booster injection three mice from each group were bled. Serum antibody levels were measured in HI tests against Jap. 305 (A_2E), X-7(F1) (A_0E), X-9 (A_2e), and X-15 (Eq E) viruses. Enzyme-inhibition titers were determined with X-7(F1) (A_0E) virus, and plaque size-reducing titers were determined against X-7 (A_0E) and NWS (A_0e) viruses in clone 1-5C-4 human con-

junctival cells (Table 7). Immunization of mice with E neuraminidase did not induce production of HI antibody against any of the viruses tested except X-15 (Eq E), but it did produce serum titers of 1:400 of enzyme-inhibiting antibody against X-7(F1) enzyme. In addition, the pooled sera of mice immunized with purified (E) enzyme had plaque size-reducing activity against X-7 virus (A_0E) but not against NWS (A_0e)—a virus that differs from the X-7 virus recombinant only in having a different neuraminidase protein (e).

When groups of mice similarly immunized were challenged with Jap. 305 (A_2E) virus, the results given in Table 8 were obtained. Mice immunized with (E) neuraminidase had considerably lower pulmonary virus titers than control mice 2 and 4 days after Jap. 305 virus challenge; they had less extensive lung lesions 7 days after challenge.

In addition, enzyme (E) immunized and unimmunized mice were challenged with X-7(F1) (A_0E), X-15 (Eq E), NWS (A_0e), and X-9 (A_2e) viruses. Titers of pulmonary virus 3 days after challenge are given in Table 9. Significant reductions of pulmonary virus titers were observed when mice immunized with (E) neuraminidase were challenged with viruses that contain A_2 (E) enzyme [X-7(F1), X-15] but not when they were challenged with viruses that have an antigenically different neuraminidase protein, e.g., (NWS, X-9).

Thus, immunization of mice with isolated A_2 neuraminidase (E) resulted in enzyme-inhibiting serum antibody to A_2 neuraminidase and, despite the lack of serum antibody reactive in hemagglutination-inhibition tests with any of the challenge viruses except X-15, led to significant protection against challenge infection with viruses containing A_2 neuraminidase.

Passive immunization of mice with rabbit antiserum to purified E neuraminidase. Mice were immunized passively by intraperitoneal injection of 0.5 ml of a 1:10 dilution of rabbit antiserum to X-7(F1) enzyme (106-day bleeding) or with a 1:2 dilution of rabbit anti-X-9 (A_2e) antiserum; control mice were injected with normal rabbit serum. All but five mice from each group were challenged 24 hr later by an aerosol infection with Jap. 305 (A_2E) virus. As part of the same experiment, previously unimmunized animals were injected with the same antisera

TABLE 6. *Effect of immunization of mice with two UV-inactivated recombinant viruses (A_0e and A_0E) on pulmonary virus titers, lung lesions, and HI antibody response following challenge with Jap. 305 (A_2E) virus*

Immunization[a]	Pulmonary virus titer			Lung lesions (%)		HI antibody titer[b] (Jap. 305 A_2E)				
	Day 2	Day 4	Day 6	Day 4	Day 6	Day 0	Day 2	Day 4	Day 6	Day 8
X-3 (A_0e)	7.9	7.2	6.5	8	45	<1:8	<1:8	<1:8	<1:8	1:16
X-7 (A_0E)	7.3	6.3	4.6	3	10	<1:8	<1:8	<1:8[c]	<1:14[d]	1:38
Saline	8.3	7.7	6.6	20	50	<1:8	<1:8	<1:8	<1:8	1:16

[a] UV-irradiated virus (hemagglutination titer = 1:64 per 0.1 ml); 0.2 ml intraperitoneally at weekly intervals; challenge, 7 days after third injection.
[b] EID_{50} $\log_{10}$, five animals in each group.
[c] Two of five sera positive for HI at 1:8 dilution.
[d] Five of five sera positive for HI at dilution of 1:8 or greater.

TABLE 7. *HI, enzyme inhibiting (EI), and plaque size-reducing (PSR) antibody response in mice immunized with purified A_2 neuraminidase*

Immunization[a]	HI antibody titer				EI titer[b]	PSR titer[c]	
	Jap. 305 (A_2E)	X-9 (A_2e)	X-7(F1) (A_0E)	X-15 (Eq E)	X-7(F1) (A_0E)	X-7(A_0E)	NWS (A_0e)
Saline–adjuvant	<1:8	<1:8	<1:8	<1:8	<1:10	<1:200	<1:200
Enzyme–adjuvant	<1:8	<1:8	<1:8	1:128	1:400	1:8,000	<1:200

[a] Subcutaneous injection, 0.3 ml of a mixture of equal parts of saline and complete Freund's adjuvant or of a 1:16 dilution of purified X-7(F1) enzyme and Freund's adjuvant, 47 and 7 days prior to bleeding.
[b] Dilution of serum inhibiting 50% of neuraminidase activity of X-7(F1) virus.
[c] Final dilution of antiserum incorporated in agar overlay effective in reducing plaque size.

TABLE 8. *Effect of immunization with isolated A_2 neuraminidase on influenza Jap. 305 (A_2E) virus infection of mice*

Immunization[a]	Pulmonary virus titers[b]		Lung lesions (%), day 7
	Day 2	Day 4	
Saline-adjuvant.....	7.8	7.2	44.0
Enzyme-adjuvant[a].....	5.5	<3.7	5.0

[a] Subcutaneous injection as in Table 7.
[b] EID_{50}, log_{10}, five animals in each group.

TABLE 9. *Effect of immunization with purified A_2 neuraminidase on challenge infections with influenza viruses which do and do not contain A_2 enzyme (E)*

Immunization[a]	Pulmonary virus titers 72 hrs after challenge[b]			
	X-7 (F1) (A_0E)	NWS (A_0e)	X-9 (A_2e)	X-15 (Eq E)
Saline-adjuvant.....	5.1	6.8	6.0	6.7
Enzyme-adjuvant...	<2.1	7.0	6.4	4.4

[a] Subcutaneous injection as in Table 7.
[b] EID_{50}, log_{10}, five animals in each group.

intranasally, 24 hr after infection with Jap. 305 virus. Uninfected cohort mice of all these immunization groups were used to obtain serum and bronchial washings for antibody determinations 48 hr after infection of their cohorts. Pulmonary virus titers were determined in the infected mice 2 and 4 days after infection, and lung lesions were assessed on the seventh day of infection. The experimental design is given schematically in Table 10, and the results appear in Table 11. Mice immunized intraperitoneally with rabbit anti-A_2 neuraminidase (Anti-E) serum 24 hr before infection or intranasally 24 hr after infection, although lacking antibody in their sera or bronchial secretions to the A_2 hemagglutinin of the challenge Jap. 305 (A_2E) virus were partially protected. They had considerably lower pulmonary virus titers 2 and 4 days after infection and far less extensive lung lesions 7 days after infection than their respective controls; they demonstrated a greater degree of protection than mice passively immunized intraperitoneally or intranasally with X-9 antiserum, despite the presence of low levels of HI antibody against the challenge virus in the serum or bronchial washings of the X-9 immunized groups.

DISCUSSION

Immunity in influenza has long been correlated with the presence of HI antibody directed against hemagglutinin (3). Such antibody can neutralize the virus as can antibody to the dissociated hemagglutinin (2). Recent studies suggest that influenza virus neuraminidase represents only 7 to 15% of the total virus protein (10), that the ratio of hemagglutinin to neuraminidase sites on the virion surface is 2:1 (R. G. Webster, W. G. Laver, and E. D. Kilbourne, J. Gen. Virol., *in press*), and that neuraminidase probably occupies no more than 25% of the virus surface (R. G. Webster, W. G. Laver, and E. D. Kilbourne, J. Gen. Virol., *in press*). Perhaps, related to these facts, antibody to the enzyme is nonneutralizing and modifies infection by its effects on the release and yield of virus from cells (1, 7, 14, 16; R. G. Webster, W. G. Laver, and E. D. Kilbourne, J. Gen. Virol., *in press*). Therefore, it is remarkable that neuraminidase antibody is as effective as it has been shown to be. In in vitro infection this effect is readily measurable by the limitation of the extension of plaque radius (5) in the continued presence of antiserum specific for the neuraminidase component of the virus.

The present in vivo studies demonstrate that antibody specific for viral neuraminidase appears in the blood of mice following pulmonary infection with influenza viruses. Neuraminidase antibody is demonstrable in high titer in mice even following infection with influenza A_0/NWS, a virus whose enzyme is extremely unstable at 37 C (11*a*). Neuraminidase antibody is produced, therefore, not merely as a consequence of injection of rabbits with large doses of intact, preformed virus but also as the result of replication of virus in the lungs following aerosol infection of mice with approximately 100 ID_{50}. Preliminary studies of natural infection of man have demonstrated four- to eightfold increases in enzyme-inhibiting antibody to A_2 neuraminidase in paired sera of patients in the epidemic of 1967–1968 (E. D. Kilbourne, W. N. Christenson, and M. Sande, J. Virol., *in press*).

Our studies show that the induction of anti-neuraminidase immunity in mice, whether achieved by passive transfer of preformed antibody, by parenteral injection of enzyme, or by infection, can profoundly influence the course of subsequent influenza virus infection. Infection of mice with intact viruses (Table 4) results in almost as much protection against challenge with a virus containing homologous neuraminidase as is observed following challenge with a virus containing homologous hemagglutinin. We do not

suggest that immunity after infection or artificial immunization is as much the consequence of neuraminidase antibody as of hemagglutinin antibody but rather that under certain circumstances antineuraminidase immunity may provide significant additional protection. For example, at borderline levels of HI antibody the additional effects of neuraminidase antibody may be crucial. Thus, in earlier experiments, immunization of mice by infection with X-7 (A_0E) virus, avirulent for mice, led to production of low levels of HI (anti-A_0) antibody. However, equal (and partial)

TABLE 10. *Passive immunization of mice intraperitoneally (ip) and intranasally (in) with rabbit antiserum to X-7(F1) neuraminidase and with antiserum to X-9(A_2e) virus*, EXPERIMENTAL DESIGN

Group no.	Day					
	−1	0	1	2	4	7
1	Anti-E[a] ip	A_2 infection	—	Pulmonary virus	Pulmonary virus	Lung lesions
1a	Anti-E ip	—	—	Antibody	—	—
2	Anti-X-9[b] ip	A_2 infection	—	Pulmonary virus titer	Pulmonary virus titer	Lung lesions
2a	Anti-X-9 ip	—	—	Antibody	—	—
3	Normal rabbit serum (NRS) ip	A_2 infection	—	Pulmonary virus titer	Pulmonary virus titer	Lung lesions
3a	NRS ip	—	—	Antibody	—	—
4	—	A_2 infection	Anti-E[c] in	Pulmonary virus titer	Pulmonary virus titer	Lung lesions
4a	—	—	Anti-E in	Antibody	—	—
5	—	A_2 infection	Anti-X-9[c] in	Pulmonary virus titer	Pulmonary virus titer	Lung lesions
5a	—	—	Anti-X-9 in	Antibody	—	—
6	—	A_2 infection	NRS in	Pulmonary virus titer	Pulmonary virus titer	Lung lesions
6a	—	—	NRS in	Antibody	—	—

[a] Intraperitoneal inoculation 24 hr before infection, 0.5 ml of a 1:10 dilution of 106-day bleeding from rabbit immunized with X-7(F1) neuraminidase (enzyme-inhibiting titer, 1:4,000).

[b] Intraperitoneal inoculation 24 hr before infection, 0.5 ml of a 1:2 dilution of serum from a rabbit immunized with X-9 virus (A_2e) (HI titer vs. X-9 virus 1:256).

[c] Intranasal administration of 0.05 ml.

TABLE 11. *Effect of passive immunization of mice intraperitoneally (ip) and intranasally (in) with rabbit antiserum to purified A_2 neuraminidase and to A_2 hemagglutinin on infection of mice with influenza A_2/Jap. 305 virus infection*

Immunization[a]	Antibody titers (day 2)				Pulmonary virus titers[d]		Lung lesions (day 7)
	HI (Jap. 305)		EI[c]				
	Serum	BW[b]	Serum	BW	Day 2	Day 4	
							%
Anti-enzyme ip	<1:8	<1:8	1:200	<1:4	5.1	4.8	0
Anti-X-9(A_2e) ip	1:32	<1:8	<1:10	<1:4	6.6	5.5	9
Normal rabbit (NRS) serum ip	<1:8	<1:8	<1:10	<1:4	7.2	6.9	67
Anti-enzyme in	<1:8	<1:8	<1:10	1:16	5.7	5.7	17
Anti-X-9(A_2e) in	<1:8	1:16	<1:10	<1:4	6.7	6.6	44
NRS in	<1:8	<1:8	<1:10	<1:4	8.2	7.3	63

[a] See Table 10 for dose and time of inoculation.

[b] Bronchial wash fluid.

[c] Dilution of serum or bronchial wash fluid inhibiting enzyme activity of X-7(F1) virus by 50%.

[d] EID_{50}, $\log_{10}$, five animals in each group.

reduction of pulmonary virus titer occurred following subsequent challenge with NWS (A_0e) and Jap. 305 (A_2E) viruses, but refractoriness to reinfection with homologous X-7 virus was complete (9).

The results of immunization of mice with isolated A_2 neuraminidase derived from X-7(F1) virus confirm the observations of infection-induced immunity. Injection of the isolated neuraminidase stimulates the production of antibody only to A_2 neuraminidase (E) (7) so that the marked inhibition of virus replication observed after challenge with viruses containing A_2 neuraminidase represents the specific protective effect of antineuraminidase immunity.

The greater protection to A_2E (Jap. 305) challenge observed in mice immunized passively with A_2 neuraminidase antiserum (anti-E) compared to that observed in mice immunized passively with X-9 (anti-A_2e) antiserum does not provide evidence that the neuraminidase antibody is *more* active than the hemagglutinin antibody. No attempt was made in this experiment to inject equivalent quantities of the two antibody molecules. This experiment does provide additional evidence, however, that immunity to infection with influenza A viruses is not exclusively a function of the titers of hemagglutinin antibody in serum or respiratory secretions. The observation that mice with antibody to neuraminidase, although lacking antibody to hemagglutinin, were more protected than mice with low levels of antibody specific to the hemagglutinin component of the challenge virus suggests that in the circumstances of this experimental design neuraminidase antibody titers provide a more accurate assessment of the level of immunity than hemagglutinating inhibiting antibody titers.

Antibody to the viral neuraminidase may have an additional effect on influenza virus infection of the intact mammalian host. Preliminary studies in this laboratory showed that mice immunized with isolated A_2 neuraminidase are far less able to transmit infection than mice with equal reductions of virus titer achieved by prior immunization with hemagglutinin protein. Neuraminidase antibody, therefore, may prevent not only the release of virus from the cell surface inhibiting the spread of virus to adjacent cells but also may prevent the expulsion of virus into the environment and subsequent spread of infection.

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