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Cross-Protection in Mice After Immunization with H2N2, H3N2, and Heq2Neq2 Influenza Virus Strains

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Mice were vaccinated with the influenza viruses A/Japan/57 (H2N2), A/Hong Kong/68 (H3N2), and A/Equi/Miami/63 (Heq2Neq2) and the hemagglutinin and neuraminidase recombinants derived from these viruses. After infection with the parent viruses, protection was compared with serological findings. It was found that influenza vaccine protects not only against infection with a strain identical or closely related to the vaccine strain, but against heterologous strains as well. Vaccination with Hong Kong/68 and its neuraminidase recombinant resulted in a heterologous neuraminidase inhibition titer against Japan/57 and in a protection against infection with Japan/57. By contrast, after vaccination with Japan/57 and its neuraminidase recombinant, no relevant heterologous neuraminidase inhibition titer against Hong Kong/68 was observed, whereas a protection against infection with Hong Kong/68 did exist. A cross-protection between Hong Kong/68 and Miami/63, but no relationship in the hemagglutination or neuraminidase inhibition tests, was established in the preinfection sera. A one-way antigenic relationship between these viruses was confirmed by the rise of hemagglutinin or neuraminidase antibodies against Hong Kong/68 in the postinfection sera. No cross-protection or serological relationship existed between Miami/63 and Japan/57. Besides the hemagglutinin and neuraminidase, a third factor, the "mouse-protecting antigen," was considered to contribute to the protection obtained. According to the protection observed, the mouse-protecting antigen of Hong Kong/68 virus is related to that of Japan/57 as well as Miami/63 virus. The mouse-protecting antigens of both Japan/57 and Miami/63 are related to that of Hong Kong/68.

Antibodies directed against the hemagglutinin (HA) and neuraminidase (NA) antigens of the influenza A viruses play a role in the protection against infection by this virus (13, 24, 30, 38, 39). Viruses with altered components, which are selected by the presence of antibody in the world population, are responsible for the regular occurrence of influenza epidemics. Although a relationship has been established between the antibody titer against the HA component in the serum and protection (11, 13, 39), there are a few exceptions to this rule (10, 22, 28). Anti-NA antibodies seem to be incapable of preventing infection, but do appear to inhibit the spread of infection (4, 27, 30, 31, 33, 36).

Sero-epidemiological investigations of Masurel and Mulder (23) suggested that around 1900 the influenza virus A/Equi/Miami/63 (Heq2Neq2) or an antigenically related virus may have caused human influenza epidemics. Masurel concluded that around 1968, 10 years after the first appearance of the H2N2 virus, the Heq2Neq2 or a related virus would become epidemic in human populations (17-19). After the antigenic shift in 1968, it was found that an antigenic relation existed between the "new" virus A/Hong Kong/68 (H3N2) and the A/Japan/57 (H2N2) and A/Equi/Miami/63 (Heq2Neq2) viruses (16, 20, 21, 37). Masurel et al. (22) studied the relationship between these three viruses in mice experiments and observed the existence of a "mouse-protecting antigen" (MPA) responsible for the cross-protection found. In other animal experiments, no complete correlation was present between circulating antibody and protection (10, 14, 35).

In the present study the hemagglutination inhibition (HI) and neuraminidase inhibition (NI) antibody response in mice to vaccination with parent virus of the influenza strains A/Japan/305/57 (H2N2) (Japan/57), A/Hong Kong/1/68 (H3N2) (Hong Kong/68), and A/Equi/Miami/1/63 (Heq2Neq2) (Miami/63) and the recombinants that contain either the HA or NA component of these strains is compared to the protection rate obtained against

homologous and cross-infections with the parent viruses Japan/57, Hong Kong/68, or Miami/63.

MATERIALS AND METHODS

Virus. The influenza A strains used in this study were: (i) A/Japan/305/57 (H2N2) egg passage (E)5; ferret passage (F)3; mouse passage (M)50; E25 and the recombinants: X15Japan (Heq1N2^J) E2M12E2 = A/Equi/Praha/1/56 (Heq1) × A/Japan/57 (N2) and X9F1 (H2N1) E2M28E3 = A/Japan/57 (H2) × A/NWS/33 (N1); (ii) A/Hong Kong/1/68 (H3N2) monkey kidney passage (MK)2; E3M26E11 and the recombinants: X15HK (Heq1N2^H) E20M17E3 = A/Equi/Praha/1/56 (Heq1) × A/Hong Kong/1/68 (N2) and (H3N1) E4M30E3 = A/Aichi/2/68 (H3) × A/Bel/42 (N1); (iii) A/Equi/Miami/1/63 (Heq2Neq2) E8M29E4 and the recombinants: (Heq1Neq2) E2M18E5 = A/Equi/Praha/1/56 (Heq1) × A/Equi/Miami/1/63 (Neq2) and (Heq2Neq1) E2M20E4 = A/Equi/Miami/1/63 (Heq2) × A/Equi/Praha/1/56 (Neq1). The N2 component derived from A/Japan/305/57 virus is marked by the symbol (^{J}); that derived from A/Hong Kong/1/68 virus is indicated by (^{H}). The recombinants were supplied by G. C. Schild, World Influenza Centre, London, and E. D. Kilbourne, Mt. Sinai School of Medicine, City University of New York, New York.

After mouse adaptation, the virus was grown in the allantoic cavity of 11-day-old embryonated chicken eggs. Virus preparations, concentrated and purified according to Schild et al. (29), were used for vaccines and serology.

Immunization and infection of mice. Groups of Swiss mice with a starting weight of 14 to 16 g were vaccinated with nine viruses: three parent influenza A viruses and six recombinants. On day 0 the mice were immunized by separate intramuscular vaccinations with 0.1 ml of incomplete Freund adjuvant vaccine and 0.1 ml of aqueous vaccine, both containing 100 HA units. After 21 days, a booster of 0.1 ml of aqueous vaccine was administered. Three weeks after revaccination, 20 mice of each group were challenged with Japan virus, 20 with Miami virus, and 20 with Hong Kong virus, intranasally under light ether anesthesia with 0.06 ml of freshly collected allantoic fluid (50× the 50% lethal dose). At the same time, control groups of 20 or 21 nonimmunized mice were infected with each strain. The vaccinations and infections were carried out separately for each virus strain, and the mice were maintained isolated by group. Blood was obtained by means of heart puncture. After vaccination, serum was collected and pooled by vaccine group to determine the HA and NA antibody titers. Ten days after challenge or infection, blood was collected from each group of surviving mice, and lung effects were registered.

Determination of infection. Lung effects and mortality in mice were determined as described by Masurel et al. (22). Lung effects within each immunized group were compared with those of the nonimmunized mice 10 days after infection and expressed as: percent lung effects = $[(n_1 \cdot 1 + n_2 \cdot 2 + n_3 \cdot 3 + n_4 \cdot 4)$ of the immunized challenged mice$/(n_1 \cdot 1 + n_2 \cdot 2 + n_3 \cdot 3 + n_4 \cdot 4)$ of the nonimmunized infected mice$] \times 100$, where n_1 = percentage of mice with 1 to 10% of the lung surface affected (value: 1); n_2 = percentage of mice with 10 to 40% of the lung surface affected (value: 2); n_3 = percentage of mice with 40 to 80% of the lung surface affected (value: 3); and n_4 = percentage of mice with 80 to 100% of the lung surface affected (value: 4).

Mortality within one challenge group was compared with that of the infected control mice of that challenge group and expressed as: percent mortality = (percentage of immunized challenged dead mice/percentage of nonimmunized infected dead mice) × 100.

Less than 60% mortality was chosen as significant for protection. The mortality after infection of the control group with the mouse-adapted Japan/57 and Miami/63 viruses was 100%, and with the mouse-adapted Hong Kong/68 virus it was 65%. The incomplete mortality after Hong Kong infection was also found by Herzberg (8–10) and Masurel et al. (22).

HI and NI test. The mouse sera were examined in the HI test according to Masurel (20) and in the NI test by the method of Aymard-Henry et al. (1). HI and NI titers were expressed as the reciprocal of the highest serum dilutions giving 50% inhibition. A titer rise of >3 was chosen as significant for these pooled sera. All serological tests were done twice.

RESULTS

Tables 1 to 3 present the mortality and lung effect percentages of the vaccinated mice after challenge with Japan/57, Hong Kong/68, and Miami/63 virus, respectively, as well as the HA and NA antibody titers detected at the time of challenge and 10 days afterwards.

Table 1 shows that mice immunized with Japan/57 virus or its recombinants were protected against mortality (0 to 5%) after challenge with Japan/57, although 31% lung effects were registered in mice immunized with Heq1N2^J. In sera from mice immunized with the Heq1N2^J recombinant, no HA antibodies directed against Japan/57 were observed. Mice immunized with Hong Kong/68 or the recombinant Heq1N2^H showed 55 and 42% mortality after challenge with Japan/57, respectively. Only NA antibodies cross-reacting to Japan/57 were measured in the sera of these two groups. No serological relationship with no protection against Japan/57 was found in mice immunized with the H3N1 recombinant. Mice immunized with Miami/63 or its recombinants were not protected against the challenge with Japan/57, and no serological cross-reaction was observed. The NI antibody titers against Japan/57 virus in pre- and post-infection sera were significantly higher in the protected groups than in the unprotected ones.

Table 2 shows that mortality after challenge with Hong Kong/68 virus was 8 and 31% for mice vaccinated with Japan/57 virus and the Heq1N2^J recombinant, respectively. Nine out of 20 animals immunized with the H2N1 recombi-

TABLE 1. *Protection after and HI and NI titers pre- and postchallenge with A/Japan/305/57 (H2N2) virus in mice immunized with Japan, Hong Kong, and Miami viruses and their recombinants*[a]

Mice immunized with	10 days after challenge[b]		No. survived out of 20	A/Japan/305/57 (H2N2)				A/Hong Kong/1/68 (H3N2)				A/Equi/Miami/1/63 (Heq2Neq2)			
				HI titer		NI titer		HI titer		NI titer		HI titer		NI titer	
	M	TL		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Hong Kong															
H3N1	100	100	1	<9	270	<30	ND[c]	330	135	40	ND	<9	<9	<30	ND
Miami															
Heq2Neq2	89	90	3	<9	540	<30	<30	<9	<9	<30	<30	70	24	360	440
Heq2Neq1	99	100	1[d]	<9	ND	<30	ND	<9	ND	<30	ND	<9	ND	<30	ND
Heq1Neq2	95	96	2	<9	1,100	<30	<30	<9	<9	<30	<30	<9	<9	890	420
Japan															
H2N2	0	5	20	3,250	2,150	1,800	1,290	<9	<9	35	<30	<9	<9	<30	<30
H2N1	0	16	20	680	2,150	30	80	<9	<9	<30	<30	<9	<9	<30	<30
Heq1N2[J]	5	31	19	<9	135	280	480	<9	<9	<30	<30	<9	<9	<30	<30
Hong Kong															
H3N2	55	78	9	<9	480	105	550	1,100	540	540	810	<9	<9	<30	<30
Heq1N2[H]	42	65	12	<9	190	230	120	<9	<9	120	510	<9	<9	<30	<30
Controls			1												

[a] Pre, Prechallenge; Post, postchallenge.
[b] M, Percentage of mortality; TL, percentage of lung effects of the total group.
[c] ND, Not done.
[d] 17 vaccinated mice were used.

TABLE 2. *Protection after and HI and NI titers pre- and postchallenge with A/Hong Kong/1/68 (H3N2) virus in mice immunized with Japan, Hong Kong, and Miami viruses and their recombinants*[a]

Mice immunized with	10 days after challenge[b]		No. survived out of 20	A/Japan/305/57 (H2N2)				A/Hong Kong/1/68 (H3N2)				E/Equi/Miami/1/63 (Heq2Neq2)			
				HI titer		NI titer		HI titer		NI titer		HI titer		NI titer	
	M	TL		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Japan															
H2N1	69	83	11	680	970	30	50	<9	135	<30	70	<9	<9	<30	<30
H2N2	8	25	19	3,250	2,150	1,800	2,500	<9	135	35	160	<9	<9	<30	<30
Heq1N2[J]	31	42	15	<9	<9	280	180	<9	70	<30	70	<9	<9	<30	<30
Hong Kong															
H3N2	0	7	20	<9	<9	105	65	1,100	1,100	540	690	<9	<9	<30	<30
H3N1	0	29	20	<9	<9	<30	<30	330	3,850	40	580	<9	<9	<30	<30
Heq1N2[H]	8	41	19	<9	<9	230	130	<9	135	120	1,400	<9	<9	<30	<30
Miami															
Heq2Neq2	15	41	18	<9	<9	<30	<30	<9	135	<30	200	70	30	360	330
Heq2Neq1	15	36	18	<9	<9	<30	<30	<9	270	<30	360	<9	<9	<30	<30
Heq1Neq2	30	55	16	<9	<9	<30	<30	<9	70	<30	<30	<9	<9	890	370
Controls			7												

[a] Pre, Prechallenge; Post, postchallenge.
[b] M, Percentage of mortality; TL, percentage of lung effects of the total group.

nant died after Hong Kong/68 infection. HA and NA antibodies against Hong Kong/68 virus were not detectable in preinfection serum of this vaccination group. After challenge with Hong Kong/68 virus, mice immunized with this parent strain or the recombinants showed a mortality of 0 to 8%, whereas the percentages of lung effects ranged from 7 to 41. Mice vaccinated with Miami/63 virus or its recombinants showed a mortality of 15 to 30% after challenge with Hong Kong/68 virus. The NI titer after challenge found against Hong Kong/68 was 200 and

TABLE 3. *Protection after and HI and NI titers pre- and postchallenge with A/Equi/Miami/1/63 (Heq2Neq2) virus in mice immunized with Japan, Hong Kong, and Miami viruses and their recombinants*[a]

Mice immunized with	10 days after challenge[b]		No. survived out of 20	A/Japan/305/57 (H2N2)				A/Hong Kong/1/68 (H3N2)				A/Equi/Miami/1/63 (Heq2Neq2)			
				HI titer		NI titer		HI titer		NI titer		HI titer		NI titer	
	M	TL		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Japan															
H2N2	95	96	2	3,250	1,100	1,800	1,150	<9	<9	35	30	<9	<9	<30	35
H2N1	84	90	4	680	190	30	<30	<9	<9	<30	<30	<9	<9	<30	65
Heq1N2^J	89	91	3	<9	<9	280	85	<9	<9	<30	<30	<9	<9	<30	115
Hong Kong															
Heq1N2^H	68	84	7	<9	<9	230	<30	<9	<9	120	210	<9	<9	<30	100
H3N2	0	14	20	<9	<9	105	50	1,100	2,150	540	680	<9	<9	<30	110
H3N1	58	66	9	<9	<9	<30	<30	330	1,100	40	540	<9	<9	<30	<30
Miami															
Heq2Neq2	32	46	14	<9	<9	<30	<30	<9	<9	<30	40	70	35	360	440
Heq2Neq1	0	20	20	<9	<9	<30	<30	<9	<9	<30	50	<9	12	<30	65
Heq1Neq2	42	50	12	<9	<9	<30	<30	<9	<9	<30	<30	<9	<9	890	1,300
Controls			1[c]												

[a] Pre, Prechallenge; Post, postchallenge.
[b] M, Percentage of mortality; TL, percentage of lung effects of the total group.
[c] 21 control mice were used.

360 in mice immunized with Miami/63 virus and the Heq2Neq1 recombinant, respectively, and negative in mice immunized with the Heq1Neq2 recombinant. A titer rise (≥12) of the HA and NA antibodies after challenge with Hong Kong/68 was found in mice immunized with the H3N1 recombinant against Hong Kong/68.

Table 3 shows no cross-reaction in the HI and NI tests between Miami/63 on the one hand and the Hong Kong/68 and Japan/57 strains on the other hand in preinfection sera. No cross-protection was found between Miami/63 and Japan/57 (see also Table 1). Mice immunized with Hong Kong/68 were protected against a challenge with Miami/63 virus (0% mortality). The percentage of mortality in mice vaccinated with the Hong Kong/68 recombinants H3N1 and Heq1N2^H was 58 and 68%, respectively, after challenge with Miami/63 virus. Of mice immunized with Miami/63 or its recombinants, 0 to 42% died after challenge with the parental strain. The HI antibody titer against Miami/63 virus was low after immunization with the parent strain (70) and negative after vaccination with the Heq2Neq1 recombinant. After infection with Miami/63, the NI antibody response against this virus remained very low. In sera of mice vaccinated with the H3N1 recombinant, the HI titer against Hong Kong/68 increased from 330 to 1,100 and the NI titer increased from 40 to 540 after challenge with Miami/63 virus.

DISCUSSION

The results of this investigation show that, in contrast to the orthodox opinion, the protection of immunized mice against challenge with a mouse-lethal influenza strain is not necessarily correlated to the antibody titers measured before challenge against the HA or NA component of the challenge virus. These findings confirm those of Herzberg (10) and Masurel et al. (22), who also found a poor correlation between antibody titers and protection.

The relationship between the NAs of Japan/57 and Hong Kong/68 virus established by Baars et al. (2), Dowdle et al. (5), and Tumova and Easterday (37) was found in this study only after immunization with Hong Kong/68 virus and the Heq1N2^H recombinant. This result of partial antigenic relationship resembles that of Luzyanina et al. (15) and Drescher and Desselberger (7), who have distinguished three subgroups of N2 by means of the NI test and photometric antibody concentration units test since 1957. The cross-protection in mice immunized with Hong Kong/68 against challenge with Japan/57 virus could be considered to be associated with NA antibodies. Mice immunized with Japan/57 virus or the Heq1N2^J recombinant were protected against challenge with Hong Kong/68 despite the nearly complete lack of heterologous antibody titers before challenge, as was also found by Herzberg (10) and Schulman and Kilbourne (32).

No complete correlation could be found between the serological cross-reaction and the cross-protection results. This finding gives reason to assume a protecting factor (MPA), as

Masurel et al. (22) suggested before. Association of a cross-protecting MPA with the NA component of Japan/57 and of Hong Kong/68 explains the failure of vaccination with the H3N1 recombinant to protect against challenge with Japan/57 virus (Table 1) and of vaccination with the H2N1 recombinant to protect against Hong Kong/68 virus (Table 2).

The one-way antigenic relationship of the HAs of Miami/63 and Hong Kong/68 virus found in other studies (6, 20, 37) could be confirmed in this study by the HI titer rise against Hong Kong/68 (330 to 1,100) after challenge with Miami/63 of mice vaccinated with the H3N1 recombinant (Table 3). An immunogenic relationship of the Heq2 component and the Hong Kong/68 virus could be suggested by NI titers in postinfection sera of mice vaccinated with the Heq2 vaccines after challenge with the Hong Kong/68 virus (Table 2).

Immunization with Hong Kong/68 or Miami/63 resulted in a protection after cross-infection of 100 and 85%, respectively, without any serological cross-reaction in the sera before challenge. Immunization with the Hong Kong/68 recombinants showed 58 to 68% mortality after challenge with Miami/63. A good protection against challenge with Hong Kong/68 virus as well as Miami/63 virus was reached by immunization with the Miami recombinants. Again, no serological cross-reaction was found in the sera taken before challenge. Even more, the Heq1Neq2 recombinant is not related to the Hong Kong/68 virus in the NI test, as was established by Tumova and Easterday (37). This lack of correlation between serological findings in the sera taken before challenge and the cross-protection found gives evidence of an MPA.

The cross-protection observed suggests a relationship between the MPAs of Hong Kong/68 and Japan/57 and of Hong Kong/68 and Miami/63. No relationship was found between Japan/57 and Miami/63 virus.

In trying to interpret the serological findings in postinfection sera and protection, one could make the following remarks. All groups protected against challenge with Japan/57 virus (Table 1) showed higher postinfection NI titers against Japan/57 than the unprotected groups. Furthermore, the MPA can be correlated to the NI titers found in the postinfection sera. Besides the serological relationship of Hong Kong/68 and Miami/63, shown by the results in Tables 2 and 3, no further cross-relationship can be found as an interpretation of the cross-protection in mice. A poor immunogenic activity of Miami/63 virus and its recombinants, especially seen in the HI antibody titers, can be of importance with regard to the impossibility of correlating serology and protection. It could be suggested that Hong Kong/68 and Miami/63 share an MPA. Antibodies against this antigen play an important role in immunity. Furthermore, it is assumed that anti-MPA antibodies are titered with a higher degree of sensitivity by means of Hong Kong/68 virus than of Miami/63 virus. This would also explain the one-way antigenic relationship between Hong Kong/68 and Miami/63 virus previously described (6, 20, 37).

Apart from humoral antibodies against the HA and NA, the immune response to other viral components (28) has to be taken into account, together with cellular and local immunity and effects of antibodies against host components. Oxford and Schild (24) and Virelizier et al. (39) found no protective antibodies against other viral components such as the matrix protein and the ribonucleoprotein. Cambridge et al. (3) and Virelizier (38) showed cellular immunity to be subordinate to the humoral defense. Zweerink et al. (40) found no differences in their study of T-cell-mediated cytotoxicity for lysis of P185 cells infected with different influenza A viruses. Shore et al. (34) and Riottot et al. (26) could detect local antibodies only for a short time after infection, up to 13 days, in ferrets and rabbits, respectively, and no booster effect after challenge was found. In contrast, Kasturi and Hannoun (12) did find a booster effect of local antibodies in rabbits after challenge.

In our results, no influence of the host components was detected, as is shown by the lack of cross-protection between the Japan/57 and Miami/63 viruses and their recombinants (Tables 1 and 3).

Taking into account the aspects mentioned before, one can test the viral components separately for their immunogenic and protecting aspects. Purity and completeness of the solitary components are of importance.

Passive immunization and vaccination with viruses adsorbed to partially related antibodies, in which recombinants with a known genetic composition (25) can be useful, could be another way of testing protection.

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