Attenuation of Pathogenicity of Fowl Plague Virus by Recombination with Other Influenza A Viruses Nonpathogenic for Fowl: Nonexclusive Dependence of Pathogenicity on Hemagglutinin and Neuraminidase of the Virus

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A number of antigenic hybrids of influenza A viruses were produced possessing either the hemagglutinin or the neuraminidase of fowl plague virus and the corresponding antigen derived from another influenza A virus. Other recombinants were obtained carrying both surface antigens of fowl plague virus but differing from the parent in certain biological properties. None of the recombinants isolated were pathogenic for adult chickens. Most recombinants obtained after crosses between reciprocal recombinants carrying both fowl plague virus surface antigens were also apathogenic for chickens. Using the same parent recombinants for double infection some of the progeny "back-recombinants" were pathogenic, whereas others were not. From these results it is concluded that the surface components do not by themselves determine the pathogenicity of influenza A viruses.

It can be expected that a virus infection evokes a disease only when functionally important cells of the host are altered by the infection. Since the cell tropism of a virus is determined primarily through the interaction between the virus surface components and their cellular receptors, it is possible that the pathogenicity of a virus is dependent upon its surface structure. Several authors have presented evidence of the polygenic nature of pathogenicity of influenza viruses (3, 5, 9, 10). Most studies, however, were performed in organisms that are not the natural host of the virus strain used. In these cases one has to take into consideration that adaptation of the virus to growth in this alien host is conditioned by factors that do not necessarily intervene when a virus strain acquires pathogenicity in its natural host. Webster et al. (20) were the only ones who showed in the natural host system that antigenic hybrids containing the hemagglutinin of fowl plague virus and either swine or turkey influenza virus neuraminidase were pathogenic for chickens. Based on these results, they suggested a correlation between pathogenicity and the surface composition of influenza A viruses. This suggestion is especially meaningful in the light of the recent finding that the cleavage of the hemagglutinin (HA) glycoprotein is a presupposition for the infectivity of influenza viruses and that cleavage is dependent on the host cell (9). In the experiments described in this report we have reinvestigated the question of whether, indeed, the hemagglutinin is the main determinant for pathogenicity of influenza viruses.

Recombinant influenza A viruses with particular desired surface antigens (17, 18), growth potential (8), or other biological characteristics (11) can be obtained by relatively simple methods, since they are formed by random reassortment of the single-stranded viral RNA fragments in doubly infected cells (for review see reference 16). In our studies we produced recombinants in vitro and in vivo between fowl plague virus, which is a highly pathogenic strain for chickens, and other influenza A viruses, which are apathogenic in these animals. These recombinants exhibited exchanged hemagglutinin or neuraminidase as well as other altered biological properties. The results show clearly that no particular combination of surface components alone is capable of specifically determining the pathogenicity of influenza A viruses and, hence, that the apparent correlation between pathogenicity and surface composition of influenza A viruses is invalid.

MATERIALS AND METHODS

Viruses. The following influenza viruses were used: plaque-purified fowl plague virus, strain Rosstock (Hav1N1) = FP., A/chick/Germany (Hav2Neq1)

= N, A/equine/Miami/1/63 (Heq2Neq2) = E, A/swine/1976/31 (Hsw1N1) = Sw, A/FM/1/47 (H1N1) = FM, A/Singapore/1/57 (H2N2) = Si, A/Japan 305/57 (H2N2) = J, and A Hong Kong/1/68 (H3N2) = Ho. The viruses were grown in the allantois of 11-day-old chicken embryos.

Cell cultures and plaque assays. Chicken embryo cell (CEC) monolayer cultures prepared from 11-day-old embryos were used 24 h after seeding at a cell density of 5×10^6 cells in plastic petri dishes of 5-cm diameter (14). Plaque assays were carried out as described (1, 9).

Production of antigenic hybrids. Antigenic hybrids containing the hemagglutinin (H) of FP virus and the neuraminidase (N) of another influenza A virus were obtained by isolating recombinants after double infection of CEC with a multiplicity of about 10 to 50 of each virus strain. The mixed virus yields were diluted and plated in CEC for plaque isolation. The agar overlay contained 2.5% of an anti-FP-neuraminidase serum prepared according to Seto and Rott (15). The antibody content of the serum used was such that under the experimental conditions plaque formation of the virus that carries the homologous neuraminidase was completely suppressed. The plaques obtained were picked after 3 days of incubation and the isolates were further purified by at least five serial plaque passages before classification according to the antigenic properties.

For the isolation of antigenic hybrids containing the neuraminidase of FP virus and the hemagglutinin from another influenza A virus, CEC were kept after double infection with culture medium containing 5 μg of trypsin/ml. It has been shown recently that all influenza A strains except FP are rendered infectious for CEC only in the presence of trypsin (9). Mixed virus yields were diluted, treated for 30 min at room temperature with chicken antiserum against influenza A/equi/Prague (Heq1/Neq1), which contains antibodies against FP virus hemagglutinin and N neuraminidase (13), and plated on CEC for plaque isolation. The agar overlay contained 10 μg of trypsin/ml (9). After five to seven serial plaque passages the isolates were tested for their antigenic properties.

Experimental animals. White leghorn chickens (8 months of age) were housed under isolation conditions. The animals were inoculated with infectious allantoic fluid (128 HA units/ml) intramuscularly. The chickens that did not die after infection were exsanguinated 14 days postinfection. For virus isolation some chickens were killed by day 3 after infection and samples of the lung, liver, spleen, and kidney were obtained. The samples were suspended in phosphate-buffered saline, pH 7.2, homogenized, and centrifuged at low speed, and the supernatants were stored in 5-ml aliquots at −80 C within 1 h after death.

Serological tests. HA titrations and hemagglutination inhibition tests were done according to standard procedures (4). Neuraminidase and neuraminidase inhibition assays using fetuin as a substrate were carried out as previously described (15).

RESULTS

Biological properties of the parent influenza A strains. Table 1 summarizes the biological properties of the influenza A strains used for these recombination experiments. FP virus is the most pathogenic strain, forming plaques in CEC without trypsin in the overlay and killing chickens or chicken embryos within 24 to 48 h after inoculation. Strain N also killed chicken embryos, but after infection with the other strains the embryos survived more than 48 h. All strains used can be differentiated serologically in their hemagglutinin and neuraminidase antigens (data not shown).

Properties of recombinants obtained after double infections in vitro. Two types of recombinants were obtained using specific antisera against the FP hemagglutinin and FP neuraminidase, respectively. It was relatively easy to

TABLE 1. *Biological properties of influenza A viruses used as parental strains for recombination*

Virus	Virus activities in embryonated eggs					
	Plaques[a]	HA units[b]	PFU/ml[b]	μg of NANA/ml[b]	Mean embryo death time (h)	Pathogenicity[c] (mean death time in days)
FP	Large, clear	1,024	3.5×10^9	275	24	+ (1.5)
N	Large, clear[d]	1,024	1.3×10^9	26.5	44	−
E	Large, clear[d]	1,024	4×10^8	500	>48	−
Sw	Small, clear[d]	512	6×10^8	41	>48	−
FM	Small, turbid[d]	512	6.5×10^8	70	>48	−
Si	Small, turbid[d]	256	5×10^8	64	>48	−
J	Small, turbid[d]	256	6×10^7	160	>48	−
Ho	Small, turbid[d]	64	4.3×10^7	51	>48	−

[a] Plaque diameter <2 mm = small, > 2 mm = large.
[b] Virus activities in allantoic fluids after inoculation of 11-day-old chicken embryos with approximately 100 PFU of virus.
[c] Pathogenicity for 8-month-old white leghorn chickens; −, no clinical symptoms
[d] Ten micrograms of trypsin per ml in agar overlay.

get recombinants with the hemagglutinin of FP and the neuraminidase of the other influenza A strains by adding anti-FP-neuraminidase serum to the agar overlay medium during the plaque tests, since only those isolates having the hemagglutinin of FP form plaques on CEC without trypsin (9).

For the isolation of recombinants containing the neuraminidase of FP and the hemagglutinin of the other strains, it was necessary to plaque the isolates in the presence of trypsin in the agar overlay. For this reason, it was not possible to have serum in the overlay medium, so the virus isolates were treated with antiserum specific for FP hemagglutinin before plaquing. In this way it was possible to specifically neutralize viruses possessing the FP hemagglutinin.

Stable recombinants were obtained only after cloning serially with at least five plaque passages.

When no antiserum was used for selection, several plaques were isolated that showed an unusual plaque morphology that was never seen in the FP wild type. The isolates contain both antigenic surface characteristics of FP.

The biological properties of all these recombinants and isolates are summarized in Table 2. It is noteworthy that all isolates had lost their pathogenicity for chickens. Furthermore, when

the recombinants were compared with their parental strains, they multiplied in embryonated eggs with virtually identical yields and similar HA titers, but extremely low neuraminidase activities. The same low neuraminidase-hemagglutinin ratio was found intracellularly.

With all recombinants carrying the hemagglutinin of FP, the mean embryo death time was longer than that of FP. With the exception of FP(H)Si(N), FP(H)E(N), FP(H)Ho(N), FP(H)Sw(N), and FP(H)FM(N), single-cycle growth curves as measured by HA titers established a retardation of virus multiplication in CEC. Also, the yields of hemagglutinin after infection of CEC with all recombinants, except for FP(H)N(N)(t), was lower than that obtained after infection with FP. For the recombinants with the neuraminidase from FP and the hemagglutinin of the other strains, the growth curves in CEC correspond fairly well to the parental strain carrying the same hemagglutinin (data not shown).

All chickens infected with the recombinants produced antibodies against the surface antigens of the respective recombinants. The hemagglutination inhibition and neuraminidase inhibition titers were in the range between 1:64 to 1:1,024. Hence, these recombinants probably multiplied in the infected birds. Antibodies against those antigens of the parental strains

TABLE 2. Biological properties of influenza A virus recombinants

Recombinants	Plaques[a]	Virus activities in embryonated eggs				
		HA units[b]	PFU/ml[b]	μg of NANA/ml[b]	Mean embryo death time (h)	Pathogenicity[c]
FP (H) N (N)	Small, clear	128	1×10^6	25.5	44	−
FP (H) N (N)	Small, turbid	1,024	5×10^8	23.5	26	−
FP (H) FM (N)	Small, clear	256	7.5×10^7	0.5	36	−
FP (H) FM (N)	Small, turbid	64	2.4×10^6	2.0	36	−
FP (H) E (N)	Small, clear	128	7×10^7	0.2	44	−
FP (H) E (N)	Small, turbid	1,024	2×10^8	43.3	32	−
FP (H) J (N)	Small, clear	512	1.2×10^8	33	40	−
FP (H) Ho (N)	Small, clear	1,024	1.5×10^8	79	32	−
FP (H) Si (N)	Small, clear	128	1.6×10^9	3.2	>48	−
FP (H) Si (N)	Large, turbid	256	5×10^8	152	48	−
FP (H) Sw (N)	Small, turbid	64	2.7×10^8	24	40	−
N (H) FP (N)	Small, clear[d]	1,024	6×10^8	294	46	−
E (H) FP (N)	Small, clear[d]	64	8×10^8	200	>48	−
Si (H) FP (N)	Small, clear[d]	128	3.3×10^6	135	>48	−
Sw (H) FP (N)	Small, clear[d]	64	1.5×10^8	93	>48	−
FP (H) FP (N) ← FM[e]	Small, clear	256	5.4×10^7	86	44	−
FP (H) FP (N) ← J[e]	Large, turbid	128	3.1×10^7	142	44	−
FP (H) FP (N) ← Sw[e]	Small, clear	128	2.2×10^8	0	40	−
FP (H) FP (N) ← Sw[e]	Large, turbid	128	3.8×10^7	52	40	−

[a-d] See Table 1.

[e] ←FM, J, Sw, Isolates obtained after mixed infection with FP and FM, J, or Sw, respectively.

that were not present in the recombinants were not detected in the sera, which is further evidence that the recombinants were genetically stable.

Crosses between reciprocal recombinants. In the following reciprocal pairs of recombinants possessing the hemagglutinin of one strain and the neuraminidase of another were crossed by double infection of CEC. The resulting progeny strains are referred to as back-recombinants. As listed in Table 3, with two pairs [FP(H)Sw(N) × Sw(H) FP(N) and PF(H)Si(N) × Si(H)FP(N)] all of the possible recombinants have been obtained, although in different yields. With two other pairs [FP(H)*N*(N) × *N*(H)FP(N) and FP(H)E(N) × E(H)FP(N)], however, a recombination to one of the parental strains [*N*(H)*N*(N) or E(H)E(N), respectively] could not be detected, although a reasonable number of plaques were tested.

Since none of the recombinants that are summarized in Table 2 were pathogenic for chickens, it was of interest to check if back-recombinants with the surface antigens of FP would regain pathogenicity. The biological properties of back-recombinants with both FP surface antigens were examined. When possible, plaques were isolated that had the same morphological appearance (large and clear) as found for the wild type. The results are summarized in Table 4. Surprisingly, out of 19 back-recombinants tested only 7 were pathogenic for chickens. All of the latter isolates had an increased mean death time in chickens compared with the wild type. It is of special interest that separate back-recombinants from the same pair showed different pathogenicities. Again, no correlation has been found between pathogenicity and any of the other biological properties tested. All chickens surviving the infection had antibodies only against the surface antigens of FP.

Crosses between reciprocal recombinants in vivo. The finding that in most cases the back-recombinants isolated after mixed infection in vitro were apathogenic could be due either to the fact that in a particular cross no pathogenic

virus could be formed or that the pathogenic particle had escaped .detection. Therefore, crosses between reciprocal recombinants in vivo were performed with those pairs where no pathogenic back-recombinants could be isolated. In these experiments, pairs with equal HA titers were mixed and injected intravenously into two chickens. After inoculation of FP(H)Sw(N) × SW(H)FP(N) and FP(H)*N*(N) × *N*(H)FP(N), respectively (see Table 4), no clinical symptoms could be observed within 14 days.

With the pair FP(H)*N*(N)(c) × *N*(H)FP(N), isolates of different organs (see Material and Methods) were also tested for biological properties. Of the four possible recombinants all except *N*(H)*N*(N) were obtained, which is in agreement with results of the crosses between reciprocal recombinants in vitro (Table 3); however, none of the isolates were found to be pathogenic for chickens.

Because of the delay implicit with in vivo mixed infection, possibly leading to an immune response or interference, a second type of in vivo selection of back-recombinants was also used. In this approach mixed infections [FP(H)-*N*(N) and *N*(H)FP(N), or FP(H)Sw(N) and Sw(H)FP(N)] were first performed in vitro, and the progeny were then inoculated into the birds. This procedure was equally unsuccessful in producing pathogenic recombinants.

DISCUSSION

Using specific antiserum against the individual surface components of influenza A viruses it is easy to obtain specific antigenic hybrids, as has already been shown by others (for reviews see references 16, 18). According to our experience at least five plaque passages were necessary to obtain stable recombinants. It is not yet known whether the frequent segregation of parental strains during the early plaque purification steps is due to the existence of heterozygotes or is a result of clump formation.

In all of the stable recombinants, hemagglutinin and neuraminidase of only one of the parental strains were found, confirming that these activities are coded on separate genome

TABLE 3. *Crosses between reciprocal recombinants in vitro*

Recombination between:	Recombinants obtained[a]			
FP(H)*N*(N) + *N*(H)FP(N)	FP(H)FP(N) (11)	FP(H)N(N) (8)	*N*(H)FP(N) (15)	*N*(H)*N*(N) (0)
FP(H)Sw(N) + Sw(H)FP(N)	FP(H)FP(N) (5)	FP(H)Sw(N) (4)	Sw(H)FP(N) (4)	Sw(H)Sw(N) (3)
FP(H)E(N) + E(H)FP(N)	FP(H)FP(N) (7)	FP(H)E(N) (10)	E(H)FP(N) (5)	E(H)E(N) (0)
FP(H)Si(N) + Si(H)FP(N)	FP(H)FP(N) (10)	FP(H)Si(N) (5)	Si(H)FP(N) (2)	Si(H)Si(N) (4)

[a] Numbers in parentheses give the number of back-recombinants obtained.

TABLE 4. *Biological properties of back-recombinants*

Recombination pairs[a]	Virus activities in embryonated eggs						
	Recombinants tested	Plaques[b]	HA units[c]	PFU ml[c]	μg of NANA/ml[c]	Mean embryo death time (h)	Pathogenicity[d] (mean death time in days)
FP(H)*N*(N)(c) × *N*(H)FP(N)	FP(H)FP(N)	Large, clear	1,024	1×10^9	240	24	−
FP(H)*N*(N) (t) × *N*(H)FP(N)	FP(H)FP(N)	Small, turbid	128	2.1×10^8	72	32	−
FP(H)E(N) (c) × E(H)FP(N)	FP(H)FP(N)	Large, clear	512	1.4×10^7	72	28	−
	FP(H)FP(N)	Large, clear	512	1×10^8	64	28	−
	FP(H)FP(N)	Large, clear	256	8×10^8	206	28	−
	FP(H)FP(N)	Large, clear	128	3.6×10^8	152	28	−
	FP(H)FP(N)	Large, clear	1,024	1.6×10^9	208	28	+ (3.5)
FP(H)E(N) (t) × E(H)FP(N)	FP(H)FP(N)	Large, clear	128	7×10^7	90	28	+ (7)
	FP(H)FP(N)	Large, clear	1,024	1.6×10^8	200	28	−
FP(H)Sw(N) × Sw(H)FP(N)	FP(H)FP(N)	Large, clear	128	1×10^9	160	28	−
	FP(H)FP(N)	Large, clear	64	3×10^8	72	28	−
	FP(H)FP(N)	Large, clear	128	2×10^9	206	28	−
FP(H)Si(N) × Si(H)FP(N)	FP(H)FP(N)	Large, clear	1,024	1.4×10^9	166	28	−
	FP(H)FP(N)	Large, clear	128	1.9×10^9	182	28	+ (6.5)
	FP(H)FP(N)	Large, clear	512	2.9×10^9	124	28	+ (>14)
FP(H)FP(N) (t) ← Sw × Sw(H)FP(N)	FP(H)FP(N)	Large, clear	64	1.8×10^9	53	28	+ (4.5)
	FP(H)FP(N)	Large, clear	128	1.9×10^9	65	28	−
	FP(H)FP(N)	Large, clear	128	2.1×10^9	58	28	+ (4)
FP(H)FP(N) (c) ← Sw × Sw(H)FP(N)	FP(H)FP(N)	Large, clear	64	4.5×10^7	50	28	+ (3.5)

[a] (c), (t) Clear or turbid plaque recombinants; ← Sw, recombinants obtained after mixed infection with FP and Sw.

[b-d] See Table 1, *a–c*.

subunits which can be reassorted freely (for review see reference 16). As far as could be determined, the hemagglutinin found in each of the recombinants corresponded in its properties to that of one or the other parent. In several of the recombinants, neuraminidase activities were lower in the virus particle as well as in the infected cells than was the case with either parent. From the low intracellular enzyme activity it can be concluded that the low neuraminidase activity of these isolates is not due to a failure of virus assembly, but rather to the

synthesis of a defective enzyme, or possibly to a reduced rate of its synthesis. In none of the crosses between reciprocal recombinants could normal neuraminidase activities be regained; hence, this property is genetically stable and might be due to a deletion as already suggested by Mowshowitz and Kilbourne (12).

All recombinants carrying the neuraminidase of FP together with hemagglutinin of another influenza A strain were not pathogenic for chickens. This is not too surprising in the light of the recent finding that the structure of the hemagglutinin determines the infectivity of influenza viruses for CEC (9). However, those recombinants possessing the FP hemagglutinin with the neuraminidase of another strain also will not be pathogenic for birds. Furthermore, recombinants (see Table 2) possessing both envelope antigens of FP, but biologically distinguishable from the wild type, were also uniformly apathogenic. The results of the crosses between reciprocal recombinants also demonstrate that most isolates with both of the surface components of FP were not pathogenic. Interestingly enough, by using the same pair of recombinants as parent in these crosses, some of the progeny isolates were pathogenic, whereas others were not. This is compatible with the idea that the nonpathogenic back-recombinants still contained one or more genes of the nonpathogenic influenza A strain. Other evidence of nonassociation of virulence with hemagglutinin-neuraminidase phenotype was demonstrated by recombinant X-31 (H3N2 in phenotype), which has enhanced mouse lung virulence in comparison to the H3N2 parental virus (8). In relation to wild-type Hong Kong virus the X-31 recombinant proved to be "semi-attenuated" for man (2). The nonlinkage of neurovirulence exclusively to viral hemagglutinin and neuraminidase in recombinants of influenza virus A/NWS was also postulated by Mayer et al. (10). These data suggest that particular surface components by themselves are insufficient; rather, some other gene product(s) must, in addition, be an essential prerequisite for pathogenicity.

These results do not necessarily contradict those obtained by Webster et al. (19, 20), who were able to isolate during recombination studies in the natural host antigenic hybrids which were pathogenic. It could be that, in animals, the few possible pathogenic recombinants produced have a selective advantage over the nonpathogenic ones. In contrast, in our in vitro experiments isolates were picked at random that differed in plaque morphology from the wild type.

Another interesting finding is that in certain crosses between reciprocal recombinants (see Table 3) one of the expected back-recombinants could not be isolated. This could mean that in certain gene (RNA fragment) combinations particles cannot be formed or, if particles are formed, they are unable to produce plaques in CEC. Potentially, genes coding for nonstructural proteins have to be considered to be important for these processes.

All of the pathogenic back-recombinants showed a higher mean death time in chickens compared with the wild type. This could indicate that, although recombination between various influenza A strains might occur in nature at a relatively high frequence, it is, however, a rare event that such a recombinant has the same or even a higher pathogenicity than the parent viruses which could compete with them. An optimal combination of genes seems to be necessary for the occurrence of new pathogenic influenza strains.

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