JOURNAL OF VIROLOGY, Dec. 1978, p. 710-716
0022-538X/78/0028-0710$02.00/0

Vol. 28, No. 3
Printed in U.S.A.

Susceptibility of Influenza A Viruses to Amantadine Is Influenced by the Gene Coding for M Protein

MICHAEL D. LUBECK, JEROME L. SCHULMAN, AND PETER PALESE*

Department of Microbiology, Mt. Sinai School of Medicine, City University of New York, New York, New York 10029

Received for publication 1 June 1978

Influenza A virus recombinants derived from "resistant" and "sensitive" parental viruses were examined for susceptibility to inhibition by amantadine. Correlation of gene constellation and amantadine susceptibility revealed that the gene coding for M protein influences sensitivity or resistance to amantadine. All recombinants which derived an M protein from an amantadine-resistant parent were found to be resistant to amantadine. All amantadine-sensitive recombinants derived an M gene from the amantadine-sensitive parent. However, a few amantadine-resistant recombinants which derived an M gene from the sensitive parent were also isolated, suggesting that the expression of amantadine sensitivity in these recombinants may be influenced by other genes.

Amantadine hydrochloride has been shown to inhibit the replication of influenza A viruses in embryonated eggs, tissue culture, experimental animals, and humans (3-5, 9; H. Wendel, Fed. Proc. 23:287, 1964). In cell culture, all influenza A viruses have been found to be sensitive. However, comparisons of different strains have provided evidence of considerable variation in amantadine sensitivity (for a review, see reference 10). The analysis of recombinant influenza A viruses generated from "sensitive" and "resistant" parents has suggested that amantadine susceptibility can be used as a genetic marker which segregates independently from the genes coding for the viral neuraminidase and hemagglutinin (2, 20).

Recent techniques employing polyacrylamide gel electrophoresis of proteins and RNAs have permitted the complete mapping of the genomes of different influenza viruses and have enabled us to identify the derivation of each gene in recombinant viruses (11, 13, 15). Furthermore, these techniques, as well as analysis of RNAs by hybridization studies, have provided a basis for correlating strain-related differences in biological properties with differences in specific genes (1, 12, 16, 17, 17a). The purpose of the present study was to employ these techniques to identify the gene(s) responsible for differences in amantadine sensitivity.

MATERIALS AND METHODS

Cells and virus. MDCK (canine kidney) cells were used for plaque assays and for the determination of virus yields in liquid overlay (13). The influenza viruses employed in this study included A/HK/8/68 (H3N2) virus (HK virus), A/PR/8/34 (HON1) virus (PR8 virus), A/NED/84/68 (H2N2) virus (NED virus), and A/WSN/33 (HON1) virus (WSN virus). Among the recombinant viruses employed in this study are several that have been described previously. These recombinants are summarized in Table 1. Additional recombinants derived from HK and PR8 viruses (R4, R6, R8, and R11) were isolated and characterized by methods which have been described previously (11, 13, 15). Recombinants R13 through R21 were derived from backcrossing recombinants R9 and R12. Recombinants R22 and R23 were derived from a backcross of R12 and R10; and R24 was derived from a backcross of R15 and R19. The gene compositions of recombinants R1 through R24 are given in Tables 2 through 5.

Plaque assays. Titrations were routinely conducted by using MDCK cell monolayers in accordance with previously described methods (19). Infected monolayers were incubated for 30 min at 37°C to allow virus adsorption before application of overlays containing different concentrations of amantadine. Comparisons of this assay system with titrations conducted by preincubating cell monolayers with amantadine for 1.5 h before infection indicated that preincubation of cells with amantadine did not alter 50% plaque reduction end points.

Chemicals. Amantadine hydrochloride (Symmetrel, 1-adamantanamine) and rimantadine hydrochloride were generously provided by C. E. Hoffmann of Pharmaceutical Research Div., E. I. du Pont de Nemours & Co., Inc., Newark, Del.

RESULTS

Amantadine sensitivities of parental viruses. Preliminary investigations of the aman-

TABLE 1. *Identification of recombinants used in previous studies*

Recombinant	Previous designation	Reference
R1	Recombinant 10	17a
R2	Recombinant 4	17a
R3	Recombinant 1	17a
R5	#1	15
R7	Recombinant 8	17a
R9	#2	15
R10	#2	11
R12	Recombinant 12	17a
RV-5	RV-5	14
RV-6	RV-6	14
RVII-1	RVII-1	14
RVII-2	RVII-2	14
RVII-3	RVII-3	14
RVII-4	RVII-4	14

tadine sensitivity of PR8, HK, NED, and WSN viruses (viruses which had been mapped previously) were conducted by titrating each of them in the presence of different concentrations of amantadine in an agar overlay. With PR8 and WSN viruses, 50% reduction of plaque number was observed at amantadine concentrations of approximately 25 μg/ml (Fig. 1). In contrast, both HK and NED viruses were about 100-fold more sensitive to inhibition by amantadine (50% reduction of plaque number at approximately 0.2 μg of amantadine per ml; Fig. 1). It should be noted that a few small turbid NED virus plaques were observed at amantadine concentrations which were completely inhibitory for HK virus (2.7 to 24.3 μg/ml).

Relationship of hemagglutinin and neuraminidase to amantadine resistance in recombinants derived from HK virus and PR8 virus. The data shown in Fig. 1 indicated that HK and PR8 viruses were sufficiently different in their amantadine sensitivity to permit analysis of the amantadine resistance of genetically defined recombinants derived from these two viruses. Recombinant viruses in which only the hemagglutinin and/or viral neuraminidase genes were exchanged were first examined for sensitivity to amantadine (Table 2). Recombinant 1 (R1) derives all of its genes from the HK virus parent with the exception of the hemagglutinin gene, which is derived from PR8 virus. This recombinant was as sensitive to amantadine as the HK virus parent. The amantadine resistance of R2, a "reciprocal recombinant" deriving only its hemagglutinin gene from the HK virus parent, was identical to that of PR8 virus. Thus, for these two recombinant viruses, amantadine resistance was shown to be not exclusively related to the viral hemagglutinin.

The gene coding for the viral neuraminidase was similarly shown to be not exclusively associated with amantadine sensitivity by the analysis of R3 (Table 2). This recombinant derives its neuraminidase from HK virus and all other genes from PR8 virus. The amantadine resistance of R3 was found to be similar to that of the PR8 virus parent. R4, a recombinant deriving both surface glycoproteins from the HK virus parent and all other genes from PR8 virus, was similarly found to be resistant to inhibition by amantadine. The data obtained when these four recombinants were used are consistent with the findings of Tuckova et al. (20) and Appleyard (2) which suggest that amantadine resistance is not linked exclusively to either or both of the surface glycoproteins.

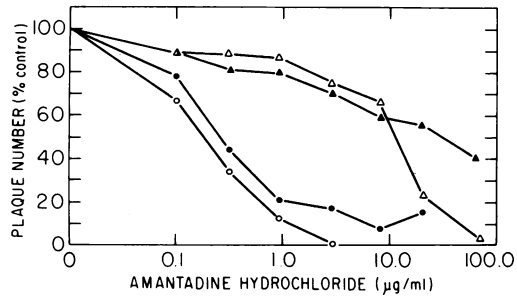

FIG. 1. *Inhibition of influenza A virus plaque formation by amantadine hydrochloride. Symbols: △, PR8 virus; ▲, WSN virus; ○, HK virus; and ●, NED virus. Control dishes contained 40 to 100 plaques per dish.*

TABLE 2. *Relationship of amantadine susceptibility and glycoproteins in recombinants derived from PR8 and HK viruses*

Virus	Gene derivation[a]			Amantadine susceptibility[b] (μg/ml)
	HA	NA	P1, P2, P3, NP, M, NS	
HK	H	H	H	0.10 (S)
PR8	P	P	P	24 (R)
R1	P	H	H	0.10 (S)
R2	H	P	P	11 (R)
R3	P	H	P	20 (R)
R4	H	H	P	24 (R)

[a] The derivation of genes for all viruses was established by electrophoresis in urea-polyacrylamide gels (see text). Proteins derived from A/HK/8/68 and A/PR/8/34 viruses are designated H and P, respectively.

[b] Amantadine susceptibility was determined by 50% reduction in plaque number in the presence of amantadine. S, Sensitive (50% reduction in plaque number at 0.1 to 0.6 μg of amantadine per ml); R, resistant (50% reduction in plaque number at 8.1 to 24.3 μg of amantadine per ml).

Influence of genes coding for NP, M, NS, and P proteins on amantadine resistance. A number of other recombinant viruses derived from HK and PR8 viruses were examined for sensitivity to amantadine (Table 3). In each instance the amantadine susceptibility of each recombinant resembled that of one or the other of the parental viruses. Examination of the gene derivation of the three amantadine-sensitive recombinants (R5, R6, and R7) indicates that they possess only two genes in common, the genes coding for P1 and M proteins, suggesting that either or both genes are needed to confer amantadine sensitivity. Examination of the gene constellation of five resistant recombinants demonstrates that four recombinants (R8, R9, R10, and R11) derive the gene for P1 protein from the amantadine-sensitive HK virus parent, indicating that the P1 gene is most likely not exclusively associated with amantadine sensitivity. Furthermore, all recombinants which derive the gene coding for M protein from PR8 virus were found to be resistant. In particular, R11, which derives the gene coding for M protein from PR8 virus and all other genes from HK virus, was found to be resistant. This analysis of data presented in Tables 2 and 3 provides strong evidence linking amantadine resistance to the gene coding for M protein. It should be noted, however, that an exception to this linkage pattern is found in R12. This recombinant derives the gene coding for M protein from HK virus but is significantly more resistant to amantadine than is HK virus (50% plaque reduction at 12 μg of amantadine per ml).

Analyses of these recombinants using rimantadine, an amantadine derivative, demonstrated that susceptibility to rimantadine was linked in an identical manner to the gene coding for M protein. Although these recombinants were found to have the same relative sensitivities, the absolute concentration of rimantadine needed for 50% plaque reduction was about five times less than that needed for amantadine.

Analysis of recombinants derived from R12. To determine whether the amantadine resistance of R12 was due to a mutation (possibly in the M gene) or reflected a particular combination of genes, backcrosses were made of R12 and two other amantadine-resistant viruses, R9 and R10 (Table 3). Among the recombinant viruses obtained from these two mixtures, eight recombinants which derived the gene for M protein from PR8 virus were isolated and found to be resistant to amantadine (only recombinants with new gene compositions, R20 and R21, are shown in Table 4). Conversely, eight of nine recombinants which derived an HK M gene from the amantadine-resistant R12 parent were found to be sensitive to amantadine (R13 through R18, R22, and R23; Table 4). In the case of the amantadine-resistant recombinant possessing an HK virus M gene, R19, the only gene of HK virus origin was the gene coding for the M protein. After recombination of R19 with R15 (Table 4), an amantadine-sensitive recombinant, R24, was obtained which was identical to R19 with the exception of the presence of an NA gene of HK virus origin. These results suggest that the amantadine resistance of R12 and R19 is probably not due to a mutation in the M gene but rather is due to the effects of certain other genes.

Effects of amantadine on virus yields. To exclude the possibility that assays of plaque reduction might not accurately reflect the effect of amantadine on virus replication, we compared virus yields of MDCK cell cultures infected with influenza viruses (multiplicity of infection, 0.005) in the presence of amantadine. Figure 2 demonstrates that HA yields obtained from PR8 virus-infected cells were not reduced in the presence of 25 μg of amantadine per ml. In contrast, a significant delay in the appearance of peak HA titers was observed with HK virus in the presence of the same concentration of amantadine. The virus yields obtained from two representative recombinants were also examined. R11, which was found to be resistant to amantadine when examined by the plaque reduction assay, was also found to be resistant in this system.

TABLE 3. *Association of amantadine susceptibility with the genes coding for NP, M, NS, and P proteins*

Virus	Gene derivation[a]								Amantadine susceptibility[b] (μg/ml)
	P1	P2	P3	HA	NA	NP	M	NS	
R5	H	H	H	H	H	H	H	P	0.25 (S)
R6	H	P	P	P	H	P	H	P	0.10 (S)
R7	H	H	H	P	P	P	H	P	0.30 (S)
R8	H	H	H	P	P	P	P	P	13 (R)
R9	H	H	H	P	H	H	P	H	26 (R)
R10	H	P	H	P	H	P	P	P	20 (R)
R11	H	H	H	H	H	H	P	H	23 (R)
R12	P	P	P	H	P	P	H	P	12 (R)

[a, b] See footnotes *a* and *b*, Table 2.

TABLE 4. *Relationship of amantadine susceptibility and gene composition of recombinants derived from R12*

Virus	Gene derivation[a]								Amantadine susceptibility[b] (μg/ml)
	P1	P2	P3	HA	NA	NP	M	NS	
R13	H	H	H	P	P	H	H	H	0.10 (S)
R14	H	H	H	P	P	H	H	P	0.25 (S)
R15	P	P	P	H	H	P	H	P	0.20 (S)
R16	H	H	P	H	H	P	H	P	0.20 (S)
R17	H	P	H	P	P	H	H	P	0.25 (S)
R18	NI[c]	NI	NI	H	H	P	H	P	0.20 (S)
R19	P	P	P	P	P	P	H	P	18 (R)
R20	H	P	P	H	H	H	P	P	18 (R)
R21	H	H	H	P	P	H	P	H	24 (R)
R22	H	P	P	P	P	P	H	P	0.6 (S)
R23	H	P	P	H	H	P	H	P	0.1 (S)
R24	P	P	P	P	H	P	H	P	0.2 (S)

[a, b] See footnotes *a* and *b*, Table 2.
[c] NI, Not identified.

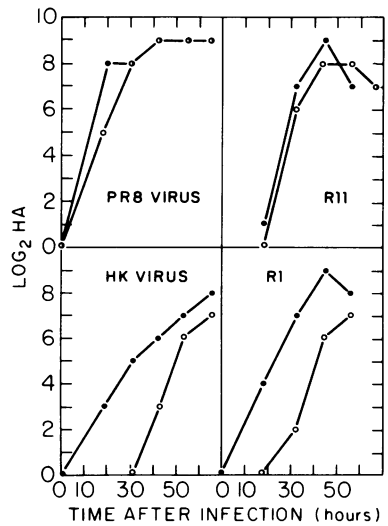

FIG. 2. *Virus yields (HA) of parental and recombinant viruses in the presence and absence of amantadine. Monolayers of MDCK cells were infected at multiplicity of infection of 0.005, and liquid overlays containing 1.0 μg of trypsin per ml were applied after an adsorption period of 30 min. Symbols: ●, no amantadine; ○, 25 μg of amantadine per ml.*

Similarly, R1, which had previously been determined to be sensitive, followed the same kinetics of growth that were observed with the HK virus parent. When R12, an amantadine-resistant recombinant possessing an HK virus M protein, was examined by the same procedures, it was again found to be much more resistant to amantadine inhibition than the HK virus parent (data not shown). It should be noted that, although the onset of HA production of amantadine-sensitive viruses is delayed, amantadine-treated and control monolayers eventually yield nearly equivalent HA titers.

Amantadine sensitivity of recombinants derived from WSN and NED viruses and WSN and HK viruses. To confirm the relationship between amantadine sensitivity and the gene coding for M protein, we employed the plaque assay system to determine the amantadine sensitivity of other genetically defined viruses. Table 5 summarizes the results obtained with three recombinants derived from amantadine-resistant WSN virus and amantadine-sensitive NED virus. RVII-4, which derives only the genes coding for P1 and M proteins from NED virus, was found to be amantadine sensitive. Analysis of two amantadine-resistant recombinants, RV-5 and RV-6, also suggests that amantadine susceptibility is determined by the gene coding for M protein. Both of these amantadine-resistant recombinants derive the gene coding for M protein from the amantadine-resistant parent (WSN virus).

Similarly, analysis of recombinants derived from WSN and HK viruses (Table 6) is in accord with the hypothesis that amantadine sensitivity is influenced by the M gene. All three recombinant viruses derive only the gene coding for M protein from HK virus and are sensitive to amantadine. It should be noted that all amantadine-sensitive recombinants derived from WSN virus demonstrated the persistence of a few very small turbid plaques at high amantadine concentrations (2.1 to 24.3 μg of amantadine per ml). Furthermore, these amantadine-sensitive recombinants were less inhibited under liquid overlay medium than was the amantadine-sensitive HK virus parent (data not shown). These results suggest that in recombinants derived from crosses of HK and WSN viruses, the quantitative expression of amantadine sensitivity may be influenced to some degree by genes other than the M protein gene.

TABLE 5. *Amantadine susceptibility of recombinant viruses derived from NED and WSN viruses*

Virus	Gene derivation[a]								Amantadine susceptibility[b] (μg/ml)	
	P1	P2	P3	HA	NA	NP	M	NS		
WSN	W	W	W	W	W	W	W	W	>24	(R)
NED	N	N	N	N	N	N	N	N	0.30	(S)
RVII-4	N	W	W	W	W	W	N	W	0.25	(S)
RV-5	N	N	N	W	W	N	W	N	>24	(R)
RV-6	N	N	N	W	W	N	W	N	>24	(R)

[a] The gene derivation of these viruses has been reported previously (see text). Proteins derived from A/WSN/33 and A/NED/84/68 viruses are designated W and N, respectively.
[b] See footnote *b*, Table 2.

TABLE 6. *Amantadine susceptibility of recombinant viruses derived from HK and WSN viruses*

Virus	Gene derivation[a]								Amantadine susceptibility[b] (μg/ml)	
	P1	P2	P3	HA	NA	NP	M	NS		
WSN	W	W	W	W	W	W	W	W	>24	(R)
HK	H	H	H	H	H	H	H	H	0.10	(S)
RVII-1	W	W	W	W	W	W	H	W	0.30	(S)
RVII-2	W	W	W	W	W	W	H	W	0.30	(S)
RVII-3	W	W	W	W	W	W	H	W	0.45	(S)

[a] The gene derivations have been reported previously (see text). Proteins derived from A/HK/8/68 and A/WSN/33 viruses are designated H and W, respectively.
[b] See footnote *b*, Table 2.

Mutation of HK virus to amantadine resistance. Next, a study was undertaken to examine the frequency of emergence of amantadine-resistant HK virus variants. Two amantadine-sensitive HK virus clones were initially obtained by plaque isolation and then passaged in embryonated eggs. MDCK cells were then infected with 5×10^3 PFU/dish with each of these cloned viruses, and an agar overlay medium containing 10 μg of amantadine per ml was added. Normal-sized plaques were selected and plaqued again in the presence of 10 μg of amantadine per ml. These isolates were found to be about 100 times as resistant to amantadine as the HK virus parent. Furthermore, after four passages in embryonated eggs in the absence of amantadine, these viruses were again found to be highly resistant to amantadine. Based on the ratio of normal-sized plaques in the presence and absence of amantadine, a crude estimate was obtained for the frequency of emergence of amantadine-resistant mutants, indicating that there were four amantadine-resistant variants per 10,000 PFU. These results are slightly lower than those obtained by Appleyard, who observed the emergence of approximately 0.1% amantadine-resistant mutants in a population of amantadine-sensitive influenza A/Bel/42 virus (2).

DISCUSSION

The analysis of 24 recombinants derived from HK and PR8 viruses indicated that differences in susceptibility to amantadine are most closely associated with differences in the gene coding for M protein. Our results indicate that all recombinants deriving an M gene from PR8 virus are resistant to amantadine. Of particular interest is R11, an amantadine-resistant recombinant deriving only the M gene from PR8 virus. Comparison of the gene compositions of R8 and R7 demonstrates that these recombinants are identical with respect to the derivation of all genes with the exception of the gene coding for M protein. R8, which derives the M gene from PR8 virus, was found to be resistant, and R7, which derives the M gene from HK virus, was found to be sensitive. Identical results are obtained from comparisons of four other pairs of recombinants: R1 and R9, R3 and R24, R4 and R15, and R13 and R21 (Tables 2, 3, and 4).

Although all amantadine-sensitive recombinants were found to contain an HK virus M gene, two amantadine-resistant recombinants (R12 and R19; Tables 3 and 4) were isolated which also derived the M gene from HK virus. R19 derived all other genes from PR8 virus, and R12 derived all other genes from PR8 virus with the exception of the HA gene. These results indicate that in the presence of a preponderance of other genes derived from the resistant parent, amantadine sensitivity in recombinants containing an HK virus M gene may not be expressed. Comparison of the recombinants R19 and R24, both of which derive the M gene from HK virus and which are identical with respect to all other

genes except the NA gene (Table 4), indicates that under these conditions the HK virus neuraminidase may influence the expression of amantadine sensitivity. Comparison of other paired viruses, such as R15 and R19, similarly suggests that under certain conditions the HK virus neuraminidase may influence the expression of amantadine sensitivity. On the other hand, comparison of recombinants R12 and R19 suggests that, under similar conditions, the HK virus hemagglutinin most likely does not influence amantadine sensitivity in recombinants with an HK virus M gene. Comparison of R19 and R22, recombinants which are identical with respect to all genes except the P1 gene, indicates that in combination with an HK virus M gene, the P1 gene also influences amantadine sensitivity. It shoud be noted that R22 appears to be significantly less sensitive to amantadine than does HK virus, indicating that susceptibility to amantadine is not always transferred as an all-or-none character. As recombinants representing all possible gene combinations are not available, it is at present not possible to identify each gene or gene combination which may influence the amantadine sensitivity of recombinants containing an HK virus M gene. However, it should be emphasized that, whatever influences other genes or combinations of genes exert, the presence of an M gene from a sensitive parent is required for amantadine sensitivity to be expressed.

The analysis of recombinants derived from crosses of WSN virus and HK or NED viruses provides additional data linking the M gene and susceptibility to amantadine. A direct association of amantadine susceptibility and the gene coding for M protein was observed in the analysis of these recombinants.

Our results suggest that amantadine resistance in R12 and R19, two recombinants containing an M protein from HK virus, is not due to mutation. However, it appears that the frequency of mutation to amantadine resistance is surprisingly high. Our data and those of Appleyard (2) suggest that amantadine-resistant mutants are found at a frequency of about 0.04 to 0.1%.

The mechanism by which amantadine inhibits influenza virus replication has not been fully elucidated. However, there is considerable evidence (5–8, 18) that amantadine inhibits an early event in the virus replication cycle, either by preventing virus penetration (5) or virus uncoating (7) or by blocking primary transcription (6). From the present results, the possibility cannot be excluded that amantadine acts by inhibiting primary transcription and that differences in M protein determine the rate at which amantadine reaches its possible target in the ribonucleoprotein complex. It would appear more likely, however, that amantadine inhibits either virus penetration or virus uncoating and that differences in the M protein directly determine the extent to which amantadine inhibits this event.

Previously, it has been shown that the capacity of recombinants for high yield in eggs requires the presence of the genes coding for M and NP proteins from high-yielding parents (17a). Thus, the relationship of strain-associated differences in amantadine susceptibility to M protein represents an additional example of differences in biological properties associated with differences in M proteins.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grants AI-11823 and AI-14053 from the National Institute of Allergy and Infectious Diseases, by grant PCM-76-11066 from the National Science Foundation, and by grant VC-234 from the American Cancer Society. M.D.L. is a Fellow of the New York Lung Association.

LITERATURE CITED

1. **Almond, J. W.** 1977. A single gene determines the host range of influenza virus. Nature (London) **270**:617–618.
2. **Appleyard, G.** 1977. Amantadine-resistance as a genetic marker for influenza viruses. J. Gen. Virol. **36**:249–255.
3. **Davies, W. L., R. R., Grunert, R. F. Haff, J. W. McGahen, R. M. Neumayer, M. Paulshock, J. L. Watts, T. R. Wood, E. L. Herman, and C. E. Hoffmann.** 1964. Antiviral activity of 1-adamantanamine (amantadine). Science **144**:862–863.
4. **Grunert, R. R., J. W. McGahen, and W. L. Davies.** 1965. The *in vivo* antiviral activity of 1-adamantanamine (amantadine). I. Prophylactic and therapeutic activity against influenza viruses. Virology **26**:262–269.
5. **Hoffmann, C. E., E. M. Neumayer, R. F. Haff, and R. A. Goldsby.** 1965. Mode of action of the antiviral activity of amantadine in tissue culture. J. Bacteriol. **90**:623–628.
6. **Kalninya, V. A., and M. K. Indulen.** 1976. Effect of amantadine derivatives on the activity of orthomyxovirus RNA-dependent RNA polymerase. Acta Virol. (Engl. Ed.) **20**:343–346.
7. **Kato, N., and H. J. Eggers.** 1969. Inhibition of uncoating of fowl plague virus by 1-adamantanamine hydrochloride. Virology **37**:632–641.
8. **Long, W. F., and J. Olusanya.** 1972. Amantadine and early events following influenza virus infection. Arch. Gesamte Virusforsch. **36**:18–22.
9. **Neumayer, E. M., R. F. Haff, and C. E. Hoffmann.** 1965. Antiviral activity of amantadine hydrochloride in tissue culture and *in ovo*. Proc. Soc. Exp. Biol. Med. **119**:393–396.
10. **Oxford, J. S.** 1977. Specific inhibitors of influenza related to the molecular biology of virus replication, p. 140–187. *In* J. S. Oxford (ed.), Chemoprophylaxis and virus infections of the respiratory tract, vol. 1. CRC Press, Cleveland, Ohio.
11. **Palese, P., M. B. Ritchey, and J. L. Schulman.** 1977. Mapping of the influenza virus genome. II. Identification of the P1, P2, and P3 genes. Virology **76**:114–121.
12. **Palese, P., M. B. Ritchey, J. L. Schulman, and E. D. Kilbourne.** 1976. Genetic composition of a high-yield-

ing influenza A virus recombinant: a vaccine strain against "swine" influenza. Science **194**:334–335.

13. **Palese, P., and J. L. Schulman.** 1976. Mapping of the influenza virus genome: identification of the hemagglutinin and the neuraminidase genes. Proc. Natl. Acad. Sci. U.S.A. **73**:2142–2146.
14. **Ritchey, M. B., and P. Palese.** 1977. Identification of the defective genes in three mutant groups of influenza virus. J. Virol. **21**:1196–1204.
15. **Ritchey, M. B., P. Palese, and J. L. Schulman.** 1976. Mapping of the influenza virus genome. III. Identification of genes coding for nucleoprotein, membrane protein, and nonstructural protein. J. Virol. **20**:307–313.
16. **Scholtissek, C., R. Rott, M. Orlich, E. Harms, and W. Rhode.** 1977. Correlation of pathogenicity and gene constellation of an influenza A virus (fowl plague). I. Exchange of a single gene. Virology **81**:74–80.
17. **Schulman, J. L., and P. Palese.** 1977. Virulence factors of influenza A viruses: WSN virus neuraminidase required for plaque production in MDBK cells. J. Virol. **24**:170–176.

17a. **Schulman, J. L., and P. Palese.** 1978. Biological properties of recombinants of influenza A/Hong Kong and A/PR8 viruses: effects of genes for matrix protein and nucleoprotein on virus yield in embryonated eggs, p. 663–674. *In* B. D. Barry and B. W. J. Mahy (ed.), Negative strand viruses and the host cell. Academic Press Inc., London.

18. **Skehel, J. J., A. J. Hay, and J. A. Armstrong.** 1977. On the mechanism of inhibition of influenza virus replication by amantadine hydrochloride. J. Gen. Virol. **38**:97–110.
19. **Tobita, K., A. Sugiura, C. Enomoto, and M. Furuyama.** 1975. Plaque assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. Med. Microbiol. Immunol. **162**:9–14.
20. **Tuckova, E., V. Vonka, A. Zavadova, and L. Kutinova.** 1973. Sensitivity to 1-adamantanamine as a marker in genetic studies with influenza viruses. J. Biol. Stand. **1**:341–346.