Acta virol. 33 : 38—42, 1989

MOLECULAR AND BIOLOGICAL PROPERTIES OF A VARIANT OF AVIAN INFLUENZA A/SEAL/MASSACHUSETTS/1/80 (H7N7(VIRUS THAT IS PATHOGENIC FOR MICE

*R. J. Podchernyaeva[1], R. G. Webster[2], V. V. Skovorodka[1], A. I. Klimov[3],

V. M. ZHDANOV[1]

[1]D. I. Ivanovsky Institute of Virology Academy of Medical Sciences, Moscow, [2]Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, 332 N. Lauderdale, P.O. Box 318, Memphis, Tennessee 38101, U.S.A. and [3]Moscow Research Institute for Viral Preparations, Moscow, U.S.S.R.

Received June 26, 1987

Summary. — A/Seal/Mass/80 influenza virus has been shown to be closely related antigenically and genetically to avian influenza H7N7 viruses, however, the virus does not replicate efficiently in avian species but does replicate in most mammals, except mice (Hinshaw *et al.*, Infect. Immun., 34, 351—361, 1981). In order to develop a model defining the molecular changes that occur during acquisition of virulence, the A/Seal/Mass/80 virus was adapted to growth in mouse lungs. The adaptation was accompanied by changes in a number of properties of the haemagglutinin as well as by changes in other genes of the virus as determined by RNA : RNA hybridization.

Key words: *influenza virus*; *variant*; *genome analysis*; *pathogenicity*; *mouse*

Introduction

A/Seal—Massachusetts/1/80 (H7N7) (A/Seal/Mass/80) influenza virus was isolated in 1979 from harbor seal (Lang *et al.*, 1981). Antigenic analysis and characterization of the RNAs (Webster *et al.*, 1981) has shown that all of the genes and gene products of this virus are closely related to avian influenza H7N7 viruses. However, biologically the virus behaves more like a mammalian strain. Thus, it was shown that A/Seal/Mass/80 virus did not replicate in avian species, but replicated in mammals (ferrets, cats, pigs) but not in mice (Hinshaw *et al.*, 1981).

In this study we have adapted the A/Seal/Mass/80 virus to replicate in the lungs of mice with the aim of developing a mode of molecular changes accompanying the appearance of pathogenic virus from a nonpathogenic parent.

* Whom requests for reprints should be addressed to

Materials and Methods

Viruses. A/Seal/Mass/80 influenza virus isolated from lungs of harbor seal (*Phoca vitulina*) (Webster *et al.*, 1981) and cloned three times at limited dilutions in embryonated eggs was used in these studies. A variant of the virus pathogenic for mice was obtained by serial passages of lung suspensions from mouse to mouse (Podchernyaeva *et al.*, 1985).

Serological tests. Haemagglutination inhibition (HI) tests were performed using polyclonal as well as monoclonal antibodies to the HA of A/Seal/Mass/80 virus (Webster *et al.*, 1982). Neuraminidase inhibition (NI) tests were done by the procedure of Aymard-Henry *et al.* (1973).

Virus neutralizing activity of monoclonal antibodies (diluted 1 : 100) was determined in tests using 100 EID_{50} of virus in chick embryos.

Pathogenicity for mice. White mice (8-10 g) were inoculated intranasally with serial dilutions of virus (0.05 ml per mouse). One mouse lethal dose (MLD_{50}) was the virus titre that caused mortality of 50 % of infected mice. Virus sensitivity to equine gamma-2-macroglobulin was assayed by HI test according to Rogers and Paulson (1983).

Genome analysis was carried out using RNA: RNA hybridization (Hay *et al.*, 1977) followed by electrophoresis of nuclease S1 ("Miles Lab" U.S.A.) treated double-stranded RNAs in 7.5 % polyacrylamide-6 mol/l urea system (Ghendon *et al.*, 1981). Exposition of gels with photosensitive film (Orwo, HS-11, G.D.R.) was carried out at 60 °C.

Results

Adaptation of A/Seal/Mass/80 virus to mice by means of serial passages of lung suspension from mouse to mouse resulted in virus replication in the lungs with titres up to 10^4 ID_{50}/0.2 ml by the third passage. After 10 passages the virus acquired pathogenic properties resulting in mice mortality when inoculated with 10^7 ID_{50}/0.2 ml of the virus. A highly pathogenic variant (M_{20}) was selected after 20 passages. Table 1 shows properties of the M_{20} variant in comparison with the initial A/Seal/Mass/80 virus. The pathogenicity of the M_{20} variant for mice was equivalent to 10^5 EID_{50} while the initial strain was completely non-pathogenic for mice. It should be noted that the acquired pathogenicity of the M_{20} variant was genetically stable and did not change during passage in chick embryos, MDCK, or CEF cells (results not shown).

Table 1. Comparative properties of A/Seal/Mass/80 virus and the pathogenic M_{20} variant[a].

Strain	Reproduction in[b] mice lungs (log_{10} ID_{50}/0.2 ml)	Pathogenicity for mice[c] (log_{10} LD_{50})	HA titre[d]	RCT (log ID_{50})[e] 28 °C	36 °C	41 °C	Sensitivity to 2-macroglobulin of horse serum
A/Seal/Mass/80	3.0	1.0	1/320	5.0	9.0	7.0	n.s.*
M_{20}	7.0	5.0	1/1280	6.0	9.0	8.0	1/64

[a] The variant was prepared by 20 passages in mouse lungs
[b] Reproduction activity in chick embryos after inoculation with lung suspension
[c] Titre of lethality for mice after intranasal inoculation
[d] HA-titer in allantoic fluid
[e] Reproduction activity in chick embryos at different temperatures
* n.s. — non sensitive

Table 2. Serological characteristics of A/Seal/Mass/80 virus and M_{20} variant

Strain	HI test								NI test with anti-A/Seal/Mass/80 polyclonal serum
	polyclonal anti-A/Seal/Mass/80 serum	monoclonal antibodies to HA of A/Seal/Mass/80							
		3/5	8/4	14/3	46/2	55/3	58/2	71/4	
A/Seal/Mass/80	640	100	6400	100	12800	12800	12800	640	270
M_{20}	640	100	1600	100	3200	6400	12800	320	270

The M_{20} variant replicated to much higher titres (10^7 EID $_{50}$/0.2 ml) in mice lungs as compared with the initial virus (10^3 EID_{50}/0.2 ml) (Table 1). The pathogenic variant was also able to replicate in eggs to higher titres at low (28 °C) and at high (41 °C) temperatures.

The sensitivity of the viruses to gamma-2-macroglobulin from horse serum differed and suggested variation in receptor-binding properties of the HA. In contrast to the initial virus, which was non-sensitive to the serum inhibitor, the M_{20} variant was inhibited to a titre of 1 : 64 (Table 1).

We compared the antigenic specificities of the haemagglutinin and neuraminidase (NA) of the initial and highly pathogenic variants of A/Seal/Mass/80 using polyclonal antisera or monoclonal antibodies to the HA of the parent virus. Only minor antigenic differences between the parent and variant strains could be detected: lower titres were found in HI tests with the M_{20} variant when tested with monoclones 8/4 and 46/2 (Table 2).

Differences between the non-pathogenic parental viruses and the pathogenic variants for mice were seen in neutralization tests with monoclonal antibodies to A/Seal/Mass/80 haemagglutinin. Table 3 shows that all the monoclonal antibodies completely neutralized the infectivity of the parent Seal virus while the M_{20} variant was not neutralized by monoclonal antibodies 8/4 and 14/3 and was only partially neutralized by the monoclone 55/3.

Since other genes may also be involved in determining the increased pathogenicity for mice, we compared the genomes of the two variants using RNA : RNA hybridization followed by electrophoresis of double-stranded RNAs in 7.5 % PAG — 6 M urea system (Hay *et al.*, 1977; Ghendon *et al.*, 1981).

Table 3. Virus neutralizing activity of monoclonal antibodies to HA of A/Seal/Mass/80 virus

Strain	Monoclonal antibodies (1 : 100)						
	3/5	8/4	14/3	46/2	55/3	58/2	71/4
A/Seal/Mass/80	0/3*	0/3	0/3	0/3	0/3	0/3	0/3
M_{20}	0/3	2/3	3/3	0/3	1/3	0/3	0/3

numerator — number of eggs with haemaglutinating activity; denominator — number of eggs
* infected

The data show (Fig. 1) that the parent A/Seal/Mass/80 strain and the mouse pathogenic variant differ not only in the genes coding for HA and NA, but also in each of the other genes as well.

Discussion

The data presented show that after serial passages of A/Seal/Mass/80 virus in mice lungs a highly pathogenic variant was selected (M_{20}). The pathogenicity of this variant was stable and did not revert after multiple passages in chick embryos, MDCK, or CEF cells.

HI tests with monoclonal antibodies to the HA of A/Seal/Mass/80 virus demonstrated only minor differences in antigenic specificity between the parental virus and the M_{20} variant. The differences in HI tests were confirmed in virus neutralization tests. The receptor-binding activity of the parent and variant viruses were different as indicated by the sensitivity to horse serum gamma-2-macroglobulin.

Thus, the data show that the adaptation of A/Seal/Mass/80 virus to mice lungs resulted in selection of variants that were highly pathogenic for this species. This adaptation is accompanied by changes in the haemagglutinin. This agrees with the concept that even single mutations in influenza virus hemagglutinin can markedly influence virus pathogenicity (Naeve *et al.*, 1984; Webster *et al.*, 1986).

The RNA: RNA hybridization studies permitted us to determine the changes not only in HA- and NA-genes but also in the genes coding for the nonglycosylated proteins. It is not possible to establish the role of these changes in pathogenicity of A/Seal/Mass/80 virus for mice; firstly the RNA: RNA hybridization technique determines both silent and coding mutations, and secondly a gene constellation rather than mutations in a particular gene can be responsible for appearance of virulent virus (Rott *et al.*, 1979). Nevertheless, the changes obtained in the genes coding for the virus polymerase complex should be specially noted. The possible role of these proteins in influenza virus pathogenicity was proposed by several authors (Bonin and Scholtissek, 1983; Skovorodka *et al.*, 1983). For example, replacement of the PI (PA) gene in A/Seal/Mass/80 virus with the PA gene from A/PR/8/34 (H1N1) resulted in reassortant which was non-pathogenic for mice (Skovorodka *et al.*, 1983). Additionally, the specific virion polymerase activity of the M_{20} variant was higher than that of the parental A/Seal/Mass/80 strain (Pravdina *et al.*, 1984, 1986).

Additional investigations of influenza viruses in different hosts and of particular mutations will be necesssary to fully elucidate the role of individual mutations in the pathogenicity of influenza viruses.

References

Aymard-Henry, M., Coleman, M. T., Dowdle, W. R. Lawer, W. G., Schild, G. C., and Webster, R. G. (1973): Influenza virus neuraminidase and neuraminidase inhibition test procedure. *Bull. Wrld. Hlth. Org.* **48**, 199–202.

Bonin, J., and Scholtissek, C. (1983): Mouse neurotropic recombinants of influenza A viruses. *Arch. Virol.* **75**, 255–268.

Ghendon, Y. Z., Klimov, A. I., Alexandrova, G. I., and Polezhaev, F. I. (1981): Analysis of genome composition and reactogenecity of recombinants of cold-adapted and virulent virus strains. *J. gen. Virol.* **53**, 215–224.

Hay, A. J., Limniczi, B., Bellamy, A. R., and Skehel, J. J. (1977): Transcription of the influenza virus genome. *Virology* **83**, 337–355.

Hinshaw, V. S., Webster, R., Easterday, B. C., and Bean, W. J. (1981): Replication of avian influenza A viruses in mammals. *Infect. Immun.* **34**, 354–361.

Lang, G., Garnon, A., and Geraci, J. R. (1981): Isolation of an influenza A virus from seals. *Arch. Virol.* **68**, 189–195.

Naeve, C. W. Hinshaw, V. S., and Webster, R. G. (1984): Mutations in the hemagglutinin receptor-binding site can change the biological properties of an influenza virus. *J. Virol.* **51**, 567–569.

Podchernyaeva, R. J., Webster, R. G., Skovorodka, V. V., Sokolova, N. N., and Zhdanov, V. M. (1985): Antigenic properties of influenza virus H7N7 variants and its recombinants (in Russian). *Vop. Virus.* **30** (**2**), 144–149.

Pravdina, N. F., Podchernyaeva, R. J., Skovorodka, V. V., Galegov, G. A., and Zhdanov, V. M. (1984): The RNA polymerase activity of pathogenic and nonpathogenic variants of influenza virus. *Vop. Virus.* **29** (**2**), 152–155.

Pravdina, N. F., Podchernyaeva, R. J., Skovorodka, V. V., and Zhdanov, V. M. (1986): Activity of virion RNA polymerase of the influenza A and B virus variants differing in their pathogenity for mice (in Russian). *Molek. Genet.* **1986**, (**10**), 29–33.

Rogers, G. N., and Paulson, J. C. (1983): Receptor determinants of human and animal influenza virus isolates: differences in receptor specifity of the H3 hemagglutinin based on species of origin. *Virology* **127**, 361–373.

Rott, R., Orlich, M., and Scholtissek, C. (1976): 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 virus. *J. Virol.* **19**, 54–60.

Skovorodka, V. V., Farashyan, V. R., Podchernyaeva, R. J., and Zhdanov, V. M. (1983): The role of individual genes for the pathogenicity of influenza virus (in Russian). *Vop. Virus.* **28** (**6**), 719–723.

Webster, R. G., Hinshaw, V. S., Been, W. J., Van Wyke, K. L., Garaci, J. K., St. Aubin, D. J., and Petursson, G. (1981): Characterization of an influenza virus from seals. *Virology* **113**, 712–724.

Webster, R. G., Hinshow, V. S., and Laver, W, G. (1982): Selection and analysis of antigenic variants of the neuraminidase of N2 influenza virus with monoclonal antibody. *Virology* **17**, 93–104.

Webster, R. G., Kawaoka, Y., and Bean, W. J. (1986): Molecular changes in A/chicken/Pennsylvania/83 (H5N2) influenza virus associated with acquisition of virulence. *Virology* **149**, 165–173.

Legend to Figure (Plate X):

Fig. 1. Genome analysis of A/Seal/Mass/80 virus and M_{20} variant CEF cell cultures were inoculated with the viruses (100 EID_{50}/cell) and incubated in the presence of cycloheximide (100 μg/ml) and ^3H-uridine (3.7 MBq/ml) for 4 hr. Complementary RNAs (cRNAs) were isolated and hybridized with an excess of virion RNA (vRNA) of either A/Seal/Mass/80 virus or the M_{20} variant. Double stranded RNAs thus obtained were treated with nuclease S1 (1000 U/ml) and electrophoresed in 7.5 % polyacrylamide gel — 6 M urea system (Hay *et al.*, 1977; Ghendon *et al.*, 1981).
c — ^3H-cRNA; v—vRNA; S — A/Seal/Mass/80; M — M_{20}; 1 — P_1; 2 — P_3; 3 — P_2; 4 — HA; 5 — NP; 6 — NA; 7 — M; 8 — NS