Acta virol. **27**: 463—469, 1983

ROLE OF GENES 4 AND 6 FOR THE EXPRESSION OF SOME BIOLOGICAL PROPERTIES OF INFLUENZA VIRUS A/PR/8/34

L. DÖHNER, B. HERRMANN, R. MENTEL, S. KRENZ

Institute for Medical Microbiology and Epidemiology, University Greifswald, 2200 Greifswald, G.D.R.

Received August 17, 1982; revised March 19, 1983

Summary. — Genetic composition and biological properties of influenza virus recombinants A/PR/8/34 (H1N1) and A/Greifswald/6/74 (H3N2) were analysed. The haemagglutinin (HA) gene of the strain A/PR/8/34 was shown an important part of the gene complex determining the virulence for mice, the yield of HA and the plaque forming capacity. The exchange of the HA gene with that of an another strain led to a drastic reduction of these properties. On the other hand, the introduction of the HA gene of A/PR/8/34 strain into the genome of an another strain, did not render the latter virulent for mice. The production of H3 HA proceeded at an about 5-fold higher extent when the H3-gene was cooperating with the genes of A/PR/8/34 in contrast to the genes of A/Greifswald/6/74.

Key words: influenza virus recombinants; genetic composition; virulence; biological properties

Introduction

Each segment of the influenza A virus genome codes for a single virus protein with exception of segments 7 and 8 which code each for two proteins, M_1 and M_2, NS_1 and NS_2, respectively (Lamb *et al.*, 1978, 1981). The exchange of one or more single stranded RNA segments between different influenza A virus strains often leads to a change of certain biological properties of the virus, e. g. host range, virulence, plaque formation capacity etc.

The comparison of genetic composition and biological behaviour of recombinants with those of parental strains may elucidate the genetic basis of the most important properties of influenza viruses. From many efforts made in this direction (Bean and Webster, 1978; Oxford *et al.*, 1978; Scholtissek, 1978; Rott *et al.*, 1978, Scholtissek *et al.*, 1979; Florent, 1980; Sugiura and Ueda, 1980) it became clear, that for most of the biological markers a certain cooperation of different genes is essential rather than the action of a certain gene. Nevertheless, exchange of single genes allows to test to which extent a certain gene of the gene complex participates in determination of virulence

or host range. According to this, we have studied how far the HA and neuraminidase (NA) genes of the strain A/PR/8/34 are connected with the yield, plaque forming capacity and virulence for mice.

Materials and Methods

Virus strains and recombinants. As parental influenza virus strains A/PR/8/34 (H1N1) and A/Greifswald/6/74 (H3N2) were used. Recombinants containing the gene 4 or genes 4 and 6 from strain A/Greifswald/6/74 were prepared according to the method of Tobita and Kilbourne (1974) and Palese and Schulman (1974) by simultaneous passaging of both parental strains in primary chick embryo cells (CEC) followed by 2 to 4-fold plaquing in CEC in the presence of 5 μg trypsin/ml and antibodies against the HA of the strain A/PR/8/34. The procedure resulted in obtaining the recombinants 1—10. For preparing the recombinants containing most of the genes of A/Greifswald/ /6/74 but the HA of A/PR/8/34, thermally inactivated strain A/PR/8/34 (Herrmann, 1978) selected in the presence of anti-A/Greifswald/6/74 serum were used. Two such recombinants (11 and 12) were isolated.

Selection of inhibitor resistant mutants. The recombinants 7 and 10 were passaged 3 times in minced chorioallantoic membrane fragments (Horvath, 1954) in the presence of 10% inactivated rabbit serum and followed by 2-fold plaque purification. Tests for inhibitor resistance were done according to Alexandrova (1962) using rabbit, guinea pig, horse, calf and human sera.

Virulence testing in mice. Pneumovirulence of the strains and recombinants was tested by intranasal inoculation of 0.05 ml virus dilutions in 20-day-old white mice. The LD_{50} was estimated by the method of Kärber (1931) using dead rates from day 3 to day 14 post-infection. Each LD_{50} value was based on 2—3 titrations using at least 10 animals for each virus dilution.

Gene composition of the recombinants was determined by hybridization of viral RNA of the parental strains with ^{3}H-labelled cRNA of the recombinants followed by S_1-nuclease treatment and electrophoresis in 4% and 7.5% polyacrylamide gels as described by Hay *et al.* (1977*a*, *b*, 1979).

Polypeptide analysis of parental strains and recombinants. CEC cultures were inoculated with concentrated virus at a multiplicity of infection about 100 EID_{50}/cell and incubated at 37 °C for 4 hr in Gey's solution lacking amino acids. Then ^{35}S-methionine was added (370 kBq per 3 cm ∅ Petri dish; specific activity 49.95 TBq/mmol; Radiochemical Centre, Amersham) and incubation continued for 30 min. The cells were solubilized and subjected to electrophoresis in 17% polyacrylamide gel according to Laemmli (1970) as modified by Skehel (1972).

Results

Properties of parental strains

Both strains, A/PR/8/34 (H1N1) and A/Greifswald/6/74 (H3N2) differed in several biological properties. The plaques, formed by the strain A/PR/8/34 in the presence of trypsin had diameters of 3—5 mm after 5 days of incubation. Those of the strain A/Greifswald/6/74 were smaller than 1 mm. Both strains differed in respect of HA production significantly. The mean HA titres in several pools of allantoic fluids from chick embryos infected with strain A/PR/8/34 were 1562, whereas those of A/Greifswald/6/74 reached only values of 96. When the amount of HA produced by A/PR/8/34 was considered 100 per cent then the yield of strain A/Greifswald/6/74 reached 6.1%. An even larger difference was to be seen in respect of virulence for mice. In the case of A/PR/8/34 we found that $10^{5.1}$ ID_{50} in eggs corresponded to 1 LD_{50} for mice. In the case of A/Greifswald/6/74 1 LD_{50} for mice contained $10^{7.9}$ eggs ID_{50}, i. e. if the virulence for mice of strain A/PR/8/34 was 100 per

Table 1. Gene composition and biological properties of the influenza virus strains A/PR/8/34, A/Greifswald/6/74, their recombinants and some of their mutants

Strain	Gene composition								Virulence for mice		Yield of HA		Plaque diameter
	P1	P2	P3	H	NP	N	M	NS	$\log \frac{ID_{50}}{LD_{50}}$	%	Titre	%	
A/PR/8/34	P	P	P	P	P	P	P	P	5.1	100	1562 ± 299**	100	large (3—5mm)
A/Greifswald/ /6/74	G	G	G	G	G	G	G	G	7.9	< 0.2	96 ± 34	6.1	small (< 1 mm)
1	P	P	P	G	P	P	P	P	8.7	< 0.2	533 ± 103	34.1	medium (1—
2	P	P	P	G	P	P	P	P	7.7	< 0.2	439 ± 88	28.1	medium 2 mm)
6	P	P	P	G	P	P	P	P	8.5	< 0.2	448 ± 74	28.7	medium
7s	P	P	P	G	P	P	P	P	7.8	< 0.2	496 ± 30	31.8	medium
7r	P	P	P	G*	P	P	P	P	6.5	3.8	619 ± 83	39.6	medium
3	P	P	P	G	P	G	P	P	8.2	< 0.2	558 ± 76	35.7	medium
4	P	P	P	G	P	G	P	P	8.4	< 0.2	568 ± 54	36.4	medium
5	P	P	P	G	P	G	P	P	8.7	< 0.2	648 ± 120	41.5	medium
8	P	P	P	G	P	G	P	P	8.6	< 0.2	619 ± 94	39.6	medium
9	P	P	P	G	P	G	P	P	7.8	< 0.2	619 ± 69	39.6	medium
10s	P	P	P	G	P	G	P	P	8.5	< 0.2	716 ± 113	45.8	medium
10r	P	P	P	G*	P	G	P	P	7.2	0.7	1027 ± 80	65.7	medium
11	G	G	G	P	G	G	P	G	8.8	< 0.2	325 ± 24	20.8	medium
12	G	G	G	P	G	G	P	G	8.8	< 0.2	345 ± 41	22.1	medium

* Gene with mutation.
** Standard deviation of the mean value.

Table 2. Sensitivity of recombinants 7s and 10s and their mutants 7r and 10r to heat stable serum inhibitors

Strain	Reciprocal HI-titres with sera of different species				
	human	horse	guinea pig	rabbit	calf
7s	160	5120	2560	640	80
7r	< 10	< 10	< 10	< 10	10
10s	80	1280	640	80	40
10r	< 10	< 10	< 10	< 10	40

cent, than that of strain A/Greifswald/6/74 was only 0.16% (Table 1). The genes of both strains differed when compared by the hybridization technique (according to Hay *et al.*, 1977*a*, *b*). The virus-specific polypeptides of these viruses produced in CEC cells and analysed by electrophoresis in 17% polyacrylamide gels revealed different migration rates of their HA, nucleoproteins and NS_1 polypeptides (Fig. 1).

Properties of recombinants

Altogether 12 recombinants were prepared from A/PR/8/34 and A/Greifswald/6/74 parental strains. The gene composition of these recombinants was analysed by the hybridization technique and by polyacrylamide gel electrophoresis (PAGE) of virus specific polypeptides. The recombinants could be divided into three groups (Table 1). Group 1 recombinants 1, 2, 6 and 7 had derived all genes from strain A/PR/8/34 except of the HA gene, which was derived from strain A/Greifswald/6/74. Group 2 with the recombinants 3, 4, 5, 8, 9 and 10 was characterized by possessing the HA and NA gene from strain A/Greifswald/6/74 and all other genes from strain A/PR/8/34. Group 3 with the recombinants 11 and 12 was characterized by having the HA gene and the matrix protein gene from strain A/PR/8/34 and all other genes from strain A/Greifswald/6/74.

With regard to the biological properties, medium sized plaques with diameters of 1—2 mm were formed by recombinants of all 3 groups. In the group 1, the HA yield as compared to A/PR/8/34 was reduced to a mean value of 30.7%. In the group 2 there was a slight but significant increase of the HA yield as compared to group 1. It reached 39.7% that of A/PR/8/34 and was 6.5-fold higher than that of A/Greifswald/6/74. This can be regarded as a result of a better cooperation between H3 and N2 than between H3 and N1. The strains 11 and 12 of the group 3 showed an about 78% reduction of the HA yield compared to that of strain A/PR/8/34.

In respect of the mice virulence, the strains of all three groups behaved alike to strain A/Greifswald/6/74 and proved to be more than 500-fold less virulent for mice than the strain A/PR/8/34.

Effect of mutations in the HA gene

In order to test, how far small changes of the HA structure may affect the biological properties of the influenza virus strains, we selected inhibitor resistant mutants of the recombinants 7 and 10 (Table 2). The presence of mutations in the HA gene were proved by hybridization according to Hay *et al.* (1979). In the other genes no mutations could be found.

The mutations in either strains resulted in an increase of HA yield as well as in a 10 to 20-fold increase of the virulence for mice. The plaque diameters were also enlarged. They measured 1.14 $\pm$ 0.25 mm and 1.85 $\pm$ 0.31 mm for the strains 7s and 7r and 1.17 $\pm$ 0.24 mm and 1.50 $\pm$ 0.37 mm for the strains 10s and 10r. The differences were proved significant.

Discussion

Our results show, that the HA gene of the strain A/PR/8/34 is an important part of the gene complex determining the virulence for mice, the HA yield and the plaque forming capacity. Its exchange by HA gene of another influenza virus strain leads to a drastic reduction of these properties. On the other hand, the introduction of HA gene of the strain A/PR/8/34 into the genome of another strain did not render this strain more virulent for mice. This means, that the HA gene is not the only factor responsible for virulence in mice.

The same conclusion can be made in respect of yield and plaque formation. These results are in accordance with those of Beare (1978) who found in his recombinants, that HA played an important role in virulence. This was also shown by Oxford *et al.* (1978) who produced a A/PR/8/34 — A/England/69 recombinant which was virulent for man and which contained all A/PR/8/34 genes except of the genes coding for glycoproteins. However, the virulence seemed to be influenced to a great extent by the cooperation of P genes with each other or with other genes (Bean and Webster, 1978; Oxford *et al.*, 1978; Rott *et al.*; 1978 Scholtissek *et al.*, 1979; Florent, 1980; Sugiura and Ueda, 1980).

Concerning the NA, we can suggest, that the genes for HA H3 and NA N2 show a better cooperation with each other and with the genes 1, 2, 3, 5, 7 and 8 of the strain A/PR/8/34 in respect of HA yields than the genes for HA H3 and NA N1. Differences in compatibility of genes for HA and NA were also found by Bean nad Webster (1978) in recombinants of the strain WSN and A/turkey/Ontario 7732/66.

We found that the production of HA H3 proceeded at an about 5-fold higher extent if the H3 gene was cooperating with the genes of A/PR/8/34 than with the genes of A/Greifswald/6/74. This underlines the importance of a special gene composition for the growth rate of influenza viruses. Nevertheless, the importance of the HA gene is also documented by the fact, that small structural changes of this gene caused by mutations can lead to significant changes of the biological properties of the virus. Similarly, Kil-

bourne (1978) and Kilbourne *et al.* (1979) found differences of growth characteristics and virulence of two influenza virus strains to be mediated by point mutations in the HA gene.

Acknowledgement. This investigation was supported by the HFR Grippe und ausgewählte Infektionskrankheiten. Part of this study was performed in the laboratory of Dr. J. J. Skehel, Division of Virology, National Institute for Medical Research, London. We thank Dr. J. J. Skehel and Dr. A. J. Hay for their generous support and helpful discussions. The excellent technical assistance of M. Tietz and M. Wolfgram is gratefully acknowledged.

References

Alexandrova, G. I. (1962): An analysis of the structure of the population of inhibitor-sensitive and inhibitor-resistant A2 influenza virus strains. *Acta virol.* **6**, 487—497.

Bean, W. J., and Webster, R. G. (1978): Phenotypic properties associated with influenza genome segments, pp. 685—692. In B. W. J. Mahy and R. D. Barry (Eds): *Negative Strand Viruses and the Host Cell.* Academic Press, London.

Beare, A. S. (1978): Recombinant influenza viruses from parents of known virulence for man, pp. 745—755. In B. W. J. Mahy and R. D. Barry (Eds): *Negative Strand Viruses and the Host Cell.* Academic Press, London.

Florent, G. (1980): Gene constellation of live influenza A vaccines. *Arch. Virol.* **64**, 171—173.

Hay, A. J., Bellamy, A. R., Abraham, G., Skehel, J. J., Brand, C. M., and Webster, R. G. (1977*a*): Procedures for characterization of the genetic material of candidate vaccine strains. *Dev. biol. Standard.* **39**, 15—24.

Hay, A. J., Lomniczi, B., Bellamy, A. R., and Skehel, J. J. 1977*b*): Transcription of the influenza virus genom. *Virology* **83**, 337—355.

Hay, A. J., Skehel, J. J., and Webster, R. G. (1979): Differentiation of the haemagglutinin genes of variant influenza viruses by RNA-RNA hybridization. *J. gen. Virol.* **45**, 245—248.

Herrmann, B. (1978): Gewinnung von Influenzavirusrekombinanten. *Arch. exp. Vet.-Med.* **32**, 455—464.

Horváth, S. (1954): A new sensitive method of the rolling drum type for influenza virus titration. *Acta microbiol. Acad. Sci. hung.* **1**, 481—494.

Kärber, G. (1931): Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch. exp. Path. Pharmak.* **162**, 480—483.

Kilbourne, E. D. (1978): Genetic dimorphism in influenza viruses: Characterization of stably associated hemagglutinin mutants differing in antigenicity and biological properties. *Proc. natn. Acad. Sci. U.S.A.* **75**, 6258—6262.

Kilbourne, E. D., McGregor, S., and Easterday, B. C. (1979): Hemagglutinin mutants of swine influenza virus differing in replication characteristic in their natural host. *Infect. Immun.* **26**, 197—201.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. *Nature (Lond.)* **227**, 680—685.

Lamb, R. A., Etkind, P. R., and Choppin, P. W. (1978): Evidence for a ninth influenza viral polypeptide. *Virology* **91**, 60—78.

Lamb, R. A., Lai, Ch. J., and Choppin, P. W. (1981): Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. *Proc. natn. Acad. Sci. U.S.A.* **78**, 4170—4174.

Oxford, J. S., McGeoch, D. J., Schild, G. C., and Beare, A. S. (1978): Analysis of virion RNA segments and polypeptides of influenza A virus recombinants of defined virulence. *Nature (Lond.)* **273**, 778—779.

Palese, P., and Schulman, J. (1974): Isolation and characterization of influenza virus recombinants with high and low neuraminidase activity. *Virology* **57**, 227—237.

Rott, R., Scholtissek, C., Klenk, H.-D., and Orlich, M. (1978): Structure and pathogenicity of influenza viruses, pp. 653—662. In B. W. J. Mahy and R. D. Barry (Eds): *Negative Strand Viruses and the Host Cell.* Academic Press, London.

Scholtissek, C. (1978): The genome of the influenza virus. *Curr. Top. Microbiol. Immunol.* **80**, 139—169.

Scholtissek, C., Vallbracht, A., Flehming, B., and Rott, R. (1979): Correlation of pathogenicity and gene constellation of influenza A viruses. II. Highly neurovirulent recombinants derived from non-neurovirulent or weakly neurovirulent parent virus strains. *Virology* **95**, 492—500.

Skehel, J. J. (1972): Polypeptide synthesis in influenza virus infected cells. *Virology* **49**, 23—36.

Sugiura, A., and Ueda, M. (1980): Neurovirulence of influenzavirus in mice. I. Neurovirulence of recombinants between virulent and avirulent virus strains. *Virology* **101**, 440—449.

Tobita, K., and Kilbourne, E. D. (1974): Genetic recombination for antigenic markers of antigenically different strains of influenza B virus. *J. Virol.* **13**, 347—352.

Explanation of Fig. 1. (Plate XLIX):

Polypeptides of the influenza virus strains A/PR/8/34 (P), A/Greifswald/6/74 (G), their recombinants 8, 7r, 7s, 10s, 10r, 6 and 12 and of the cell control (ZK). CEC in 30 mm Petri dishes were infected with approximately 100 EID_{50}/cell of the respective viruses and incubated in Gey's medium for 4 hr before pulsing for 30 min with 370 kBq per dish of ^{35}S-methionine. Labelled polypeptides were analysed as described by Skehel (1972) in 17% polyacrylamide gels.