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GENETIC RECOMBINATION BETWEEN TEMPERATURE-SENSITIVE AND WILD-TYPE INFLUENZA A VIRUS STRAINS

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Summary. — Genetic cross was performed between a temperature-sensitive (ts) mutant of influenza virus A/WSN (H0N1) which carries a ts lesion in M gene, and a wild type A/Aichi/2/68 (H3N2). Twelve clones were isolated randomly from the mixed yield in the absence of any selective procedure and they were individually examined for their ts character. In addition, structural and non-structural polypeptides of an individual clone were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) to identify from which parent each viral protein was derived. The experiments have shown that at high frequency recombination occurred with respect to every major viral protein; this is compatible with the view that recombination with influenza viruses is actually a mere exchange of RNA segments.

Key words: *influenza virus*; *temperature-sensitive mutant*; *genetic recombination*; *viral proteins*; *polyacrylamide gel electrophoresis*

Introduction

The influenza virus genome is composed of 8 discrete pieces of RNA of negative polarity, which are transcribed into monocistronic messengers (Scholtissek *et al.*, 1976; Inglis *et al.*, 1977). It has been suggested that genetic recombination of influenza viruses is an exchange of such pieces of RNA between the viruses (reviewed by Sugiura, 1975). Polyacrylamide gel electrophoresis (PAGE) of viral RNA's has definitely proved that this is really the case (Ueda *et al.*, 1978).

Tobita performed a genetic cross between influenza viruses A/WSN (H0N1) and A/Aichi/2/68 (H3N2) in clone 5C-4 cells which are permissive for both viruses, and isolated randomly a total of 191 clones from the mixed yield in the absence of any selective procedure. Frequency of recombinants for the serotypes of haemagglutinin (HA) and neuraminidase (NA) among these clones was as high as 29% (Tobita, 1971; 1972). Pairwise crosses

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between temperature-sensitive (ts) mutants of A/WSN (H0N1) belonging to different complementation groups have also revealed a high recombination frequency ranging from 1—24% (Sugiura *et al.*, 1972; Sugiura *et al.*, 1975)

Influenza virus virulent for man may become attenuated when it acquires the ts phenotype (Murphy *et al.*, 1972). Taking advantage of the characteristics that influenza viruses recombine efficiently, an instant attenuation of a wild type virus could be achieved by transfering the ts gene from a laboratory strain ts mutant to a wild type epidemic strain (Murphy *et al.*, 1972). If the ts gene to be transferred codes for one of the internal proteins of the virus particle, then a recipient could become temperature-sensitive while still retaining its surface proteins, namely HA and NA (Murphy *et al.*, 1975). This would be a particularly efficient means for the rapid preparation of an up-dated live influenza vaccine, coping with the antigenic changes occurring in nature.

These backgrounds in mind, we attempted at a genetic cross between a ts mutant of influenza A/WSN (H0N1), a group VII mutant carrying a ts defect in M gene, and a wild type A/Aichi/2/68 (H3N2) in MDCK cells which permit productive growth of both viruses. Clones were isolated from the mixed yield at random, and their phenotypes were characterized. The results of the experiments are described in the present communication.

Materials and Methods

Viruses. Influenza viruses A/Aichi/2/68 (H3N2) and ts-51, a temperature-sensitive mutant of A/WSN (H0N1), were used in the study. The ts-51, originally isolated by Sugiura *et al.* (1975), is a group VII mutant with a ts defect in M gene (Ritchey and Palese, 1977). Efficiency of plating of WSN ts-51 at 39.5 °C was 1.4×10^{-4} in MDCK cells, while that of Aichi was 0.4. The viruses were plaque cloned twice and propagated in MDCK cells at 35 °C.

Cells. MDCK cells were used throughout. The growth of the cells has been described elsewhere (Tobita *et al.*, 1975). After virus infection, the cells were maintained with a trypsin medium to facilitate the cleavage of the HA polypeptide into HA1 and HA2 (Klenk *et al.*, 1975).

Plaquing technique. MDCK cell monolayers in 6-cm plastic tissue culture dished (Falcon) were washed twice with phosphate-buffered saline supplemented with 0.2% bovine serum albumin (PBS + BA), and then 0.1 ml of the virus was inoculated per dish. After 30 min incubation at room temperature, 5 ml/dish of agar medium containing 4 μg/ml of trypsin was added and the dishes were incubated for 3 days. An equal volume of agar medium containing 0.0015% neutral red was then added, and the plaques were counted 5 hr later.

Mixed infection. MDCK cell monolayer was mixedly infected with 5 PFU/cell each of ts-51 and A/Aichi at 35 °C. At 3 hr post infection (p.i.), the culture was treated for 15 min with a mixture of anti-WSN and anti-Aichi rabbit antisera to neutralize the unpenetrated virus. After 3 washes of the cell sheet with PBS + BA, a fresh maintenance medium containing 4 μg/ml of trypsin was added and the culture was incubated at 35 °C for additional 13 hr.

Cloning of the mixed yield. The mixed yield was inoculated into MDCK cell monolayer cultures at a concentration of 10 PFU/culture. Plaques were allowed to develop without any selection. On day 3 p.i., a block of agar overlaying the plaques that were situated farther than 3 cm from other plaques were picked up, suspended in 1 ml of PBS + BA and stored at —80 °C. Twelve clones thus isolated were examined for their ts character and structural and non-structural proteins.

Labelling of infected cells. MDCK cell monolayers in 35-mm plastic tissue culture dishes (Falcon) containing 1.6×10^6 cells were infected with 10 PFU/cell of the clones propagated in MDCK cells. After 5 hr at 35 °C, medium was replaced with a methionine-free maintenance medium supplemented with 925 kBq/ml of ^{35}S-menthionine (Amersham, specific activity 7.77 GBq/mM) and the cells were labelled for 1 hr at 35 °C. At the end of the labelling period, cells were scraped

Table 1. Virus yield in mixed infection of MDCK cells

Cells infected with	Virus yield (PFU/ml)	HAN titer	PFU/HAN ratio
Aichi alone	1.3×10^8	512	2.5×10^5
WSN alone	2.2×10^8	1024	2.1×10^5
Aichi × WSN	1.6×10^8	512	3.1×10^5

* MDCK cells infected with 5 PFU/cell of Aichi alone, WSN alone or a mixture of both incubated in trypsin medium at 35 °C. The cultures were harvested at 16 hr p.i.

off from the dish, washed 3 times with PBS, and suspended in 20 μl of reticulocyte standard buffer (RSB; 10 mmol/l NaCl, 1.5 mmol/l $MgCl_2$, 10 mmol/l Tris-HCl, pH 7.4). Cells were lyzed by 3 cycles of snap freezing-thawing, and 10 μl of the lysate was subjected to PAGE.

Polyacrylamide gel electrophoresis. Ten μl of the sample was mixed with an equal volume of 2× sample buffer (0.1 mol/l Tris-HCl, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue), boiled for 2 min and applied to a 5—20% polyacrylamide gradient slab gel. The electrophoresis was performed for 16 hr at 40 V in a discontinuous tris-glycine-SDS system originally described by Laemmli (1970). After electrophoresis, the gel was stained with Coomassie brilliant blue, and dried with a Hoeffer gel drier. A Fuji x-ray film was exposed to the dried gel for 5 days and an autoradiogram was prepared.

Isotope. L-^{35}S-methionine (specific activity 7.771 GBq/mM) was purchased from Amersham.

Results

Virus yield after mixed infection

MDCK cells were mixedly infected with 5 PFU/cell each of A/WSN and A/Aichi strains; the 16 hr-yield was assayed for infectious virus by plaque assay in MDCK cells and for haemagglutination (HAN) activity using 0.5% chick red blood cells (Table 1). The infectivity titer and PFU/HAN ratio of the mixed yield almost equalled those of the yields after single infections, with WSN alone or Aichi alone, indicating that mixed infection did not favour the production of von Magnus type defective virus.

Ts character of the clones

Twelve plaques picked randomly from the mixed yield, each containing 4×10^4 PFU of the infectious virus on the average, were directly inoculated into MDCK cell monolayers and their infectivity was plaque-titrated in parallel at 35 and 39.5 °C. Efficiency of plating (EOP) was calculated by dividing the infectivity titer assayed at 39.5 °C by that assayed at 35 °C. Clones with EOP higher than 10^{-1} were taken as ts^+, and those with EOP less than 10^{-2} were taken as ts. Every clone fell into either of the two categories, and there were no intermediate cases. Of the 12 clones examined, 5 were ts and 7 were ts^+ (Table 2).

Table 2. Ts character of the clones

Virus clone		EOP at 39.5/35	ts character*
A/Aichi		0.4	ts^+
A/WSN		1.4×10^{-4}	ts
clone No.	1	4×10^{-3}	ts
	2	2×10^{-3}	ts
	3	0.3	ts^+
	4	0.1	ts^+
	5	3×10^{-3}	ts
	6	0.1	ts^+
	7	0.2	ts^+
	8	0.1	ts^+
	9	9×10^{-3}	ts
	10	0.7	ts^+
	11	6×10^{-3}	ts
	12	0.1	ts^+

* Clone with EOP higher than 10^{-1} were taken as ts^+; clones with EOP less than 10^{-2} were taken as ts.

Electrophoresis of WSN and Aichi viral proteins

MDCK cells infected with WSN or Aichi were labelled with 925 kBq/ml of ^{35}S-methionine for 1 hr since 5 hr p.i., and the polypeptides were analyzed on a 5—20% polyacrylamide gradient gel (Fig. 1). With both viruses, Pl-2, HA, NP, M and NS1 were clearly resolved. NA was undetectable in our present system. In addition, NS2 polypeptide could be identified only with Aichi but not with WSN. Both viruses showed an essentially identical gel pattern, except that the mobility of HA, NP and NS1 polypeptides of both viruses slightly differed from each other in the electrophoresis system employed (Fig. 1, Plate III).

Electrophoresis of the viral proteins of the clones

Twelve clones were infected and labelled as is described in Materials and Methods. The autoradiograph of the polypeptides of 4 clones (No. 1, 5, 7 and 11), run in parallel with the parental viruses, is shown in Fig. 2. HA, NP and NS polypeptides of each clone were identified either as WSN (W) or Aichi (A) on the basis of their electrophoretic mobility. Each polypeptide migrated to exactly the same position as that of either of the parents, and there was no ambiguity in identifying from which parent it was derived. For example, HA, NP and NS of clone No. 1 were determined as derived from W (open circle), A and A (closed circle), respectively (Fig. 2, Plate IV). The polypeptides of the remaining clones (No. 2, 3, 4, 6, 8, 9, 10, and 12) were analyzed exactly in the same way (results are summarized in Table 3).

Table 3. Gene constellation of the clones

Clone No.	Polypeptide marker			
	HA	NP	NS	M[3])
1	W[1])	A[2])	A	W
2	W	W	W	W
3	W	A	A	A
4	W	A	W	A
5	W	A	A	W
6	W	W	A	A
7	W	A	W	A
8	A	A	A	A
9	A	W	A	W
10	W	W	A	A
11	W	A	A	W
12	A	A	A	A
Frequency of A/W	3/10	8/4	9/3	7/5

[1]) A/WSN
[2]) A/Aichi
[3]) according to Table 2.

Discussion

When viral polypeptides synthesized in MDCK cells by influenza viruses A/WSN and A/Aichi were compared by electrophoresis in a 5—20% gradient polyacrylamide gel, HA, NP and NS of Aichi migrated slightly faster than the comparable polypeptides of WSN. Therefore, HA, NP and NS proteins of every clone obtained by a cross between WSN and Aichi could be traced back to either of the parents on the basis of their electrophoretic mobilities.

The M protein of both viruses migrated to exactly the same position, and therefore, could not be distinguished from each other in this manner. However since ts-51 of WSN is temperature-sensitive with a ts lesion in M gene, while Aichi is not, the fact that a clone is temperature-sensitive directly signifies that it has the M gene derived from WSN (ts-51) and not from Aichi. In this way, the source of HA, NP, M and NS proteins of each clone isolated from the mixed yield could be identified either from WSN or Aichi.

Genotypes of the clones deduced from such analyses (Table 3) revealed that the exchange of genes occurs fairly frequently between the two viruses. In fact, as far as HA, NP, M and NS genes are concerned, only 3 (No. 2, 8 and 12) out of 12 clones examined had the same genotype as either of the

parents, and remaining 9 had heterogeneous gene constellation. Frequency of recombination in our present system was thus estimated as 75% (9/12). Frequency of the allelic genes of two subtypes among the clones differed considerably from gene to gene. The M gene of WSN and Aichi occurred almost equally, but WSN predominated with respect to HA gene, while 9 out of 12 clones, or 75%, had NS of Aichi subtype (Table 3). No indication of the linkage was observed between any genes. It is not surprising, however, in view of the fact that recombination between influenza viruses is actually a random reassortment of independently replicating segments of viral RNA's.

Clone No. 9 is a recombinant which has HA, as well as NS, gene of Aichi subtype but possesses M gene of WSN origin and is therefore temperature-sensitive. Although a derivation of NA was not determined in the present study, this clone would be a candidate for a live attenuated vaccine against curently epidemic H3N2 influenza, since it is the anti-HA antibody which is mainly protective against influenza virus infection.

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