

## COMPARATIVE STUDIES ON INFLUENZA B VIRUS. II. THE EFFECT OF HOST CELLS ON BIOLOGICAL PROPERTIES AND POLYPEPTIDE COMPOSITION

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*Summary.* — The reproduction patterns of various influenza B virus strains isolated in 1970—1976 using roller cultures of MDCK cells and chick embryos (CE) were compared. The cultural and allantoic virus populations did not differ in their sensitivity to non-specific inhibitors from mammalian sera and in their reactivity with specific haemagglutinins (HA). The content of infectious virus and HA in the harvested allantoic fluids as compared to medium fluids were 94- and 8-fold higher, respectively, even though the fluids did not differ in the titres of complement-fixing (CF) antigen. The mol. weight (MW) of HA<sub>1</sub> polypeptide of the B/Len/75 virus prepared in culture was higher than that of the allantoic virus (57.5 K and 55.5 K, respectively). Under reducing conditions, the HA of the virus from culture was represented mostly by the uncleaved HA<sub>0</sub> polypeptide, while that of the allantoic virus by the HA<sub>1</sub> and HA<sub>2</sub> subunits. Under non-reducing conditions, the virus from medium fluid was found to contain glycopeptide D with MW of 90 K.

*Key words:* influenza B viruses polypeptide composition; MDCK cells; chick embryo allantoic fluid

### *Introduction*

Investigations on the relationship of propagating system to biological properties and/or composition of influenza virus populations showed no antigenic changes of virions produced in cell cultures under permissive conditions (Mogabgab *et al.*, 1961; Thibon *et al.*, 1967; Choppin, 1969; Ho *et al.*, 1976; Simanovskaya *et al.*, 1978). At the same time, the specific ratio of infectious and defective virus particles may vary depending on the cultivation system (Choppin, 1969; Ramos and Torres, 1970). The host system was shown to influence the virus glycoprotein composition (Compans *et al.*, 1970; Haslam *et al.*, 1970; Collins and Knight, 1978; Nakamura and Compans, 1978) — in particular the oligosaccharide composition of HA (Nakamura and Compans, 1979), the stability of HA in the presence of sodium

dodecylsulphate (SDS) (Downie, 1978), the content of neuraminidase (NA) (Laver, 1963) — although the mobility of individual viral polypeptides in polyacrylamide gel electrophoresis (PAGE) did not change significantly (Koblet, 1971).

The present work was aimed at comparison of influenza B viruses grown in MDCK cells and CE (amount of infectious virus, viral HA, CF antigen), of biological properties of the medium- and allantoic fluid-harvested viruses (sensitivity to inhibitors, reactivity with antibodies), as well as of polypeptide composition of influenza B viruses grown in different systems.

### *Materials and Methods*

*Viruses.* Influenza B virus strains described in the previous paper (Korchanova *et al.*, 1983) were used. The allantoic viruses (AV) included the strains passaged 7–9 times in the allantoic cavity of 11-day-old chick embryos inoculated with  $10^2$ – $10^3$  egg infectious doses (EID)<sub>50</sub>/0.2 ml each and incubated for 72 hr at temperature of 32 °C. The cultural viruses (CV) consisted of the strains grown in roller cultures of MDCK cells for 3–6 passages; the initial virus material grown in MDCK cells consisted of AV in its 7th–8th passage.

*MDCK cells.* The MDCK cell culture received from Professor Kilbourne (Mount Sinai School of Medicine, New York, U.S.A.) was grown in Eagle's medium (manufactured in the Institute of Poliomyelitis and Viral Encephalitis, Moscow, U.S.S.R.) and supplemented with 10% bovine serum in roller bottles of 1–4 l capacity (NBS, U.S.A.) rotated at 0.5 rev/min at a temperature of 37 °C. Subpassages were made every 3–4 days using 0.04% versene solution at a seed concentration of  $2.0$ – $4.0 \times 10^4$  cells/cm<sup>2</sup> of the growth surface, the volume of the growth medium comprising 12.5% of the roller bottle volume.

*Inoculation of MDCK cells.* Before inoculation, the cell monolayer was washed with phosphate-buffered saline (PBS) pH 7.2–7.4, the virus was added in a dose of 0.1–0.01 EID<sub>50</sub>/cell; after adsorption at 32 °C for 1 hr the virus was removed and maintenance medium without serum was added. The infected cultures were incubated in a roller apparatus for 72 hr at 32 °C. The methods used for titration of the haemagglutination and infectious activities of the virus were described in previous paper.

*Sera.* Immune rat sera raised to the allantoic and cultural viruses were prepared by 5 intraperitoneal inoculations of white rats with crude virus-containing allantoic ( $10^6$ – $10^8$  EID<sub>50</sub>/0.2 ml) or medium ( $10^5$ – $10^7$  EID<sub>50</sub>/0.2 ml) fluids.

*The sensitivity of viruses to non-specific inhibitors* of native and heated (56 °C, 30 min) horse, rat, mouse, guinea pig, and rabbit sera was tested by haemagglutination inhibition (HI) with 4 antigen units (AU) of virus.

*The CF antigen* was quantitated by titration in plexiglass panels by a micromethod in the cold (4 °C, 18 hr) using 1% sheep red blood cells, haemolytic serum, hyperimmune rat sera, and 1.5 units of complement, all the ingredients in a volume of 0.025 ml. The results were read after addition of the haemolytic system in a water bath at 40 °C (Smorodintsev *et al.*, 1969).

*The method used for PAGE* has been described in previous paper (Korchanova *et al.*, 1983). The carbohydrate portion of glycopolypeptides was stained with Schiff reagent (Zacharius *et al.*, 1969).

*Virus polypeptide labelling.* MDCK cells were grown on a glass surface of  $4 \times 5$  cm<sup>2</sup> washed with PBS and inoculated with virus in a dose of  $2.5 \times 10^4$  haemagglutination units (HAU) per bottle in a volume of 1 ml. After adsorption for 1 hr, the cells were washed 4–5 times with PBS (heated to 37 °C) and overlaid with 10 ml of Eagle's medium for 4 hr. Thereafter, the medium was removed, cells washed with PBS, and 10 ml aliquots of leucine- and valine-free Eagle's medium were added. After 1 hr the medium was removed and both, 1.85 MBq D,L-<sup>14</sup>C-leucine (specific activity 1061.9 MBq/mM) as well as 1.85 MBq D,L-<sup>14</sup>C-valine (specific activity 1202.5 MBq/mM) in 2 ml of leucine and valine free medium were added. After incubation for 40 min, the cells were washed 4 times with cold PBS and once with cold 0.05 mol/l Tris-HCl buffer containing 0.1 mol/l NaCl and 0.001 mol/l EDTA, pH 7.2. Finally cells were lysed with

**Table 1. Reproduction of influenza B viruses in CE and MDCK cells**

Viruses	Cultivation system			
	Chick embryos		MDCK cells	
	HA titre*	EID <sub>50</sub> /0.2 ml	HA titre*	EID <sub>50</sub> /0.2 ml
B/HK/72	16—64	6.2—7.7	8—32	5.0—6.3
B/Len/74	128—256	7.0—7.5	4—64	5.3—6.8
B/Len/75	128—1024	8.2—9.0	32—256	5.0—8.0
B/Len/76	128—1024	7.5—9.0	32—128	5.0—7.0
B/Sv/76	32—128	5.0—7.7	8—16	4.3—5.0
B/Khab/76	64—128	6.8—8.0	32	5.0—6.5
B/Msk/59	64—256	6.5—7.5	8—32	5.1—6.3
B/Lee/40	128—256	7.0—8.0	32—64	5.0—6.0

\* Dilution reciprocals.

0.5 ml of same buffer containing 2% SDS, 1 mol/l urea, and 2% dithiotreitol (DTT). Cell lysates were immediately heated at 100 °C for 3 min and frozen.

For the preparation of virions with labelled proteins, 1.85 MBq D,L-<sup>14</sup>C-leucine and 1.85 MBq D,L-<sup>14</sup>C-valine were added to the roller cultures of MDCK cells two hr after infection and left in the medium (250 ml) for 70 hr of incubation. Autoradiograms were obtained by electrophoresis of cell lysates and of labelled purified virions in 10% polyacrylamide gel. The gels were dried and exposed with roentgen film PM-1 for 10—15 days.

## Results

### *Reproduction activity of influenza virus in different cultivation systems*

The infectivity and HA activity of 8 influenza B virus strains cultivated in roller cultures of MDCK cells and in CE were compared. The virus yields did not depend on their antigenic relatedness. In both systems, B/Len/75

**Table 2. Quantitation of antigenicity of the allantoic and medium fluids for influenza B/Len/75 viruses**

Preparation	Geometric mean titres			Titre ratio	
	IV titre (EID <sub>50</sub> /0.2 ml)	HA titre	CF antigen titre	IV HA	IV CF antigen
	Cultural virus	1.7 × 10 <sup>6</sup> (16)	39* (32)	10.7 (30)	4.95 × 10 <sup>4</sup>
Allantoic virus	1.6 × 10 <sup>8</sup> (28)	315 (32)	9.2 (30)	5.07 × 10 <sup>5</sup>	1.74 × 10 <sup>7</sup>
AV/CV titre ratio	94	8.1	0.85	11.6	110.1

IV = infectious virus; in parentheses number of tests.

\* Dilution reciprocals.

Table 3. Sensitivity to inhibitors and affinity to antibodies of AV and CV

Virus strain	Cells	No. of passages	Titres of serum inhibitors								Antibody titres*	
			Native				Heated				Allan-toic	Cul-tural
			Mouse	Calf	Guinea pig	Rabbit	Mouse	Calf	Guinea pig	Rabbit		
B/HK/72	CE	9	<10**	<10	20	160	<10	<10	<10	20	160	40
	MDCK	3	<10	>10	20	160	<10	<10	<10	20	160	40
B/Len/74	CE	9	40	80	320	320	20	40	80	80	160	40
	MDCK	5	40	80	320	320	20	40	80	80	160	40
B/Len/75	CE	8	40	80	160	320	20	20	40	80	640	160
	MDCK	6	40	80	160	320	20	20	40	80	640	160
B/Len/76	CE	9	40	80	320	2560	20	40	40	320	160	40
	MDCK	6	40	80	320	2560	20	40	40	320	160	40
B/Khab/76	CE	10	<10	>10	80	80	>10	>10	10	40	160	40
	MDCK	8	>10	>10	80	80	>10	>10	10	40	160	40
M/Msk/59	CE	Many	40	80	160	320	20	40	40	80	20	<10
	MDCK	3	40	80	160	320	20	40	40	80	20	<10
B/Lee/40	CE	Many	10	10	80	80	<10	>10	10	40	20	<10
	MDCK	3	10	10	80	80	<10	>10	10	40	20	<10

\* After immunization of rats with influenza B/Len/75 virus.

\*\* Dilution reciprocals.

and B/Len/76 viruses proved to be the most growing ones; their HA titres in CE and in MDCK cells were 1024 and 128–256, respectively; their infectious activity titered 9 and 7–8 log EID<sub>50</sub>/0.2 ml, respectively. Reproduction of the other 6 viruses in MDCK cells was quite moderate (HA titres up to 64, infectious activity 4.3–6.8 log EID<sub>50</sub>/0.2 ml) (Table 1).

Successive cultivation of B/Len/75, B/Len/76, and B/Khab/76 viruses in MDCK cells revealed no significant differences in the values of their infectious activity and HA titres throughout 5–15 passages indicating a reproduction pattern requiring no preliminary adaptation. Calculation of geometric mean titres of B/Len/75 virus reproduction in MDCK cells and CE showed that AV contained 94 times as much infectious virus and 8 times as much HA as CV, although both preparations had virtually similar amounts of the CF antigen (Table 2).

Evaluation of the virus population according to the criteria of von Magnus (1954) showed that the ratio of infectious titre to HA titre was 11.6-fold higher in AV as compared with CV, and that the ratio of infectious virus titre to CFA titre was 110-fold higher in AV than in CV.

*Sensitivity to non-specific serum inhibitors of influenza viruses cultivated in different systems and their reactivity with specific antibodies*

Comparison of sensitivity to non-specific inhibitors and affinity to antibodies demonstrated no differences between AV and CV. Both viruses were resistant to inhibitors of native and heated rat and horse sera. The titres of inhibitors of mouse, calf, guinea pig, and rabbit sera were completely identical with AV and CV (Table 3). The cultivation system exerted no influence of the affinity of viruses to specific antibodies. The absolute values of HI antibody titres with AV and CV were identical.

*Comparison of the polypeptide composition of CV and AV*

Comparison of the polypeptide composition of B/Len/75 virus grown in MDCK cells and CE revealed differences in MW and per cent distribution of viral glycopolypeptides. The MW of HA<sub>1</sub> determined by PAGE in comparison with the mobility of marker proteins was 57.5 K for CV and 55.5 K for AV.

HA of CV (Fig. 1-IV) under reducing conditions was represented by un-cleaved HA<sub>0</sub> (21.2%) as well as HA<sub>1</sub> (21%) and HA<sub>2</sub> (12.8%) (Table 4). Under identical conditions, HA of AV (Fig. 1-III) was practically completely cleaved into HA<sub>1</sub> and HA<sub>2</sub> (Table 4). Under non-reducing conditions HA of AV was presented by HA<sub>0</sub> (Fig. 1-I), while CV, in addition to HA<sub>0</sub>, had another major high molecular polypeptide with MW of 90 K (Fig. 1-II).

The results of disk electrophoresis with staining of the carbohydrate portion of glycoproteins with Schiff reagent showed this component to be a glycopolypeptide (gp) which has been designated "D". Three minor high molecular polypeptides designated A, B, and C also contain carbohydrates and appear to be di- and trimers of HA and NA.

Analysis of the proportional protein and carbohydrate contents of gp D

**Table 4. Percentual content of polypeptides and carbohydrates in allantoic and cultural influenza B/Len/75 viruses**

Polypeptides	MW	Cultural virus			
		DTT <sup>+</sup>		DTT <sup>-</sup>	
		Polypeptides	Carbohydrates	Polypeptides	Carbohydrates
A	> 100	0	0	2.8 ± 0.6	3.3 ± 0.7
B	> 100	0	0	2.8 ± 0.6	8.7 ± 1.8
C	> 100	0	0	2.5 ± 0.5	12.5 ± 2.7
D	90	0	0	16.5 ± 1.0	44.0 ± 1.0
HA <sub>0</sub>	74.5	21.2 ± 1.7*	62.3 ± 1.5	26.0 ± 1.0	31.5 ± 2.1
NP	62	18.4 ± 1.3	0	18.2 ± 0.3	0
NA	57	21.0 ± 1.9	26.7 ± 2.3	0	0
HA <sub>1</sub>	57.5				
HA <sub>2</sub>	28	12.8 ± 2.7	11.0 ± 2.0	0	0
M	23	26.6 ± 3.8	0	31.2 ± 2.2	0

  

Allantoic virus					
A	> 100	0	0	3.5 ± 1.7	9.8 ± 0.8
B	> 100	0	0	6.5 ± 3.0	23.0 ± 2.3
C	0	0	0	0	0
D	0	0	0	0	0
HA <sub>0</sub>	74.5	5.7 ± 2.3	Traces	29.2 ± 4.3	67.2 ± 2.5
NP	62	19.8 ± 1.4	0	21.3 ± 1.7	0
NA	57	31.6 ± 2.5	61.5 ± 1.5	0	0
HA <sub>1</sub>	55.5				
HA <sub>2</sub>	28	17.3 ± 1.0	38.5 ± 2.5	0	0
M	23	25.6 ± 2.3	0	39.5 ± 1.0	0

DTT<sup>+</sup> = electrophoresis under reducing conditions; disintegrating buffer contained 100 mmol/l dithiothreitol and 2% β-mercaptoethanol.

DTT<sup>-</sup> = electrophoresis under non-reducing conditions; MW = molecular weight (in kilodaltons).

\* per cent of content.

showed that the protein portion comprised 16.5% of total viral proteins and the carbohydrate 44% of total viral carbohydrates. To find out whether gp D is an aggregate of smaller polypeptides or whether cells synthesize a polypeptide of similar MW, an additional study was carried out on the synthesis of virus-induced polypeptides in MDCK cells. Fig. 2 presents the results of simultaneous electrophoresis of purified cultural B/Len/75 virus labelled with D,L-<sup>14</sup>C-leucine and D,L-<sup>14</sup>C-valine (Fig. 2-III and 2-VI) and a lysate of MDCK cells infected with this virus (Fig. 2-II and 2-V). Polypeptides HA<sub>0</sub> (75 K), NP (62 K), NS (40 K) and M (23 K) could be clearly identified in the MDCK cell lysate. Identification of P proteins was difficult and no protein corresponding to gp D of the virion was found in the cells.

### Discussion

Correlations in the reproduction activity of some influenza B virus strains were found in two permissive cultivation systems: MDCK cells and CE. Our experimental material indirectly attests to a stable influenza B virus reproduction in different cultivation systems and to lack of association with antigenic origin of virus strains. The cultivation system neither exerted influence on the affinity to antibodies nor on the sensitivity to HA inhibitors of the 8 influenza B virus strains. In agreement with the data previously reported (Ho *et al.*, 1976), also their antigenic properties remained unchanged. At the same time, populations of AV contained more infectious virus and HA than those of CV, but the amounts of the CF antigen remained equal. These differences could be due to the intactness and stability of virions in the presence of different amounts of defective particles in populations cultivated in different systems.

Analysis of the polypeptide composition of the viruses showed the MW of polypeptide HA<sub>1</sub> of the cultural population of B/Len/75 virus to be higher than in the allantoic population. The dependence of MW of HA<sub>1</sub> upon the host cell system was previously shown by Schwarz *et al.* (1977) for fowl plague virus propagated in MDCK cells and chick embryo fibroblasts (59 K and 51 K, respectively). According to them, MW of HA<sub>1</sub> increased because of the increase of MW of oligosaccharide chains. To elucidate the origin of gp D, a major component present exclusively in CV and absent in AV, the virus-induced polypeptides synthesized in infected MDCK cells were analysed in PAGE. No gp D was found in the lysate of these cells which corresponds to observations of Racaniello and Palese (1979) with B/Lee/40 and B/Maryland/59 viruses. The nature of gp D requires further study.

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*Explanation of Micrographs (Plate XL):*

*Fig. 1.* Comparison of the mobility in PAGE (10% gel) of polypeptides of allantoic (I, III) and cultural (II, IV) variants of B/Len/75 virus. DTT<sup>+</sup> = reducing conditions (III, IV), DTT<sup>-</sup> = non-reducing conditions (I, II). Polypeptides: 1 – gp A, 2 – gp B, 3 – gp C, 4 – gp D, 5 – HA<sub>0</sub>, 6 – NP, 7 – HA<sub>1</sub>, 8 – HA<sub>2</sub>, 9 – M.

*Fig. 2.* Electrophoresis in 10% polyacrylamide gel of virion proteins and MDCK cell-synthesized proteins of B/Len/75 virus.

I and IV – proteins of uninfected MDCK cells, II and V – virus-induced proteins, III and VI – virion proteins. Reducing conditions (DTT<sup>+</sup>, IV–VI), non-reducing conditions (DTT<sup>-</sup>, I–III).

Polypeptides: 5 – HA<sub>0</sub>, 6 – NP, 7 – HA<sub>1</sub>, 8 – HA<sub>2</sub>, 9 – M, 10 – NS. NA is situated between NP and HA<sub>1</sub> (track VI).