DIFFERENCES BETWEEN ORIGINAL STRAINS AND THEIR MOUSE-ADAPTED VARIANTS OF HUMAN (H1) AND AVIAN (H2) INFLUENZA A VIRUSES IN THE REACTION WITH CROSS-NEUTRALIZING MONOCLONAL ANTIBODY RECOGNIZING CONFORMATIONAL EPITOPE

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Summary. – Human (H1) and avian (H2) influenza A viruses and their mouse-adapted (MA) variants were studied in radioimmunoprecipitation assay (RIPA) and infectivity neutralization test using a monoclonal antibody (MoAb) directed against a conserved antigenic epitope in the stem region of the haemagglutinin (HA) and reacting both with H1 and H2 subtypes of HA. Whereas the MA variant of avian influenza A virus differed from the originbal strain in RIPA and neutralization tests, no differences were observed between the original human strain and its MA variant, as well as between the original H1 and H2 strains.

Key words: influenza A virus; haemagglutinin; adaptation to mice; monoclonal antibody; cross-neutralizing activity; glycosylation

The HA glycoprotein of influenza A viruses, a component of viral envelope, performs two critical functions in the early stage of virus infection. Firstly, HA is responsible for the binding of the virus to receptors on the cell surface (Hirst, 1941). Secondly, it mediates membrane fusion and release of the viral genome into the cytoplasm (Huang *et al.*, 1981; Lenard *et al.*, 1981; Klenk *et al.*, 1988). HA is also one of the major proteins determining the host range and pathogenicity of influenza A viruses (Webster *et al.*, 1987). In the process of the interspecies barrier crossing, HA undergoes changes resulting from interactions with cells and organism of new host (Schild *et al.*, 1983; Robertson *et al.*, 1987). In the case of the adaptation to growth in mouse lungs it alters its antigenic structure, receptor-binding activity and molecular mass (Gitelman *et al.*, 1984, 1986; Shilov *et al.*, 1994; Lipatov *et al.*, 1995). These changes are the result of amino acid replacements which bring about a loss or acquisition of glycosylation sites in HA_1 subunit. The amino acid sequence of HA stem region and HA_2 subunit may remain conserved in the course of adaptation to mice (Gitelman *et al.*, 1986). However, Smeenk *et al.* (1994) described amino acid replacement in the stem part of HA_2 subunit of MA variant of HA resulting in increased virulence.

In this study we attempted to determine whether different origin, selection in the organism of mice and glycosylation of HA_1 subunit influence the conformation of the stem region.

Two strains of influenza A virus isolated from humans (H1 subtype) and birds (H2 subtype), and their MA variants were examined by RIPA and cytopathic effect (CPE) neutralization test using a cross-neutralizing antibody to HA.

MoAb C179 with unique properties (cross-neutralizing activity against H1 and H2 influenza A viruses), reacting with an anigenic epitope in the stem region of HA (Okuno *et al.*, 1993), was provided by Takara Shuzo Co. Ltd Biotechnology Research Laboratories, Japan, with the help of

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Abbreviations: CPE = cytopathic effect; EDTA = ethylenediamine tetraacetate; HA = haemagglutinin; MA = mouse-adapted; MoAb = monoclonal antibody; PAGE = polyacrylamide gel electrophoresis; p.i. = post infection; RIPA = radioimmunoprecipitation assay; Tris = tris-(hydroxymethyl)-aminomethane;

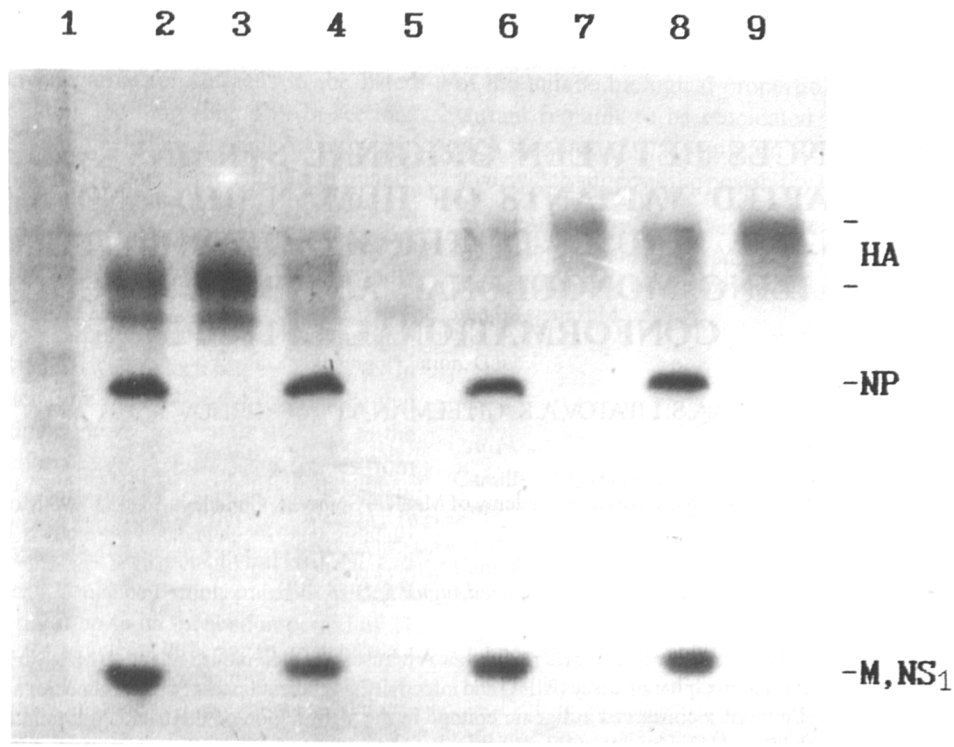

Fig. 1
RIPA of HA of the original influenza virus strains and their MA variants with MoAb C179
PAGE (12.5%) at 7.5 mA run for 16 hrs. Lane 1: mock-infected control; lanes 2, 4, 6, and 8: the lysates of the cells infected with Dk/NJ/78, Dk/NJ/78-MA, USSR/77 and USSR/77-MA, respectively; lanes 3, 5, 7, and 9: HA of Dk/NJ/78, Dk/NJ/78-MA, USSR/77 and USSR/77-MA, respectively, precipitated with MoAb C179.

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The human influenza virus strain A/USSR/90/77 (H1N1) (USSR/77) and its pneumovirulent MA variant (USSR/77-MA) were a kind gift from Dr. I.A. Rudneva, D.I. Ivanosvky Institute of Virology (for detailed characteristics of these viruses see Gitelman *et al.,* 1986; Shilov *et al.,* 1991, 1994). The avian influenza virus A/Black Duck/New Jersey/1580/78 (H2N3) (Dk/NJ/78) was obtained from Dr. R.G. Webster, St. Jude Children's Research Hospital, Memphis, Tennessee, USA. The MA variant Dk/NJ/78-MA was prepared as described previously (Lipatov *et al.,* 1995). The viruses were propagated in 9-day-old chick embryos at 37°C for 48 hrs. The virus-containing allantoic fluid was used as virus stock in the experiments.

The labelled virus-specific proteins for RIPA were obtained by routine methods. Briefly, the confluent monolayers of MDCK cells grown in Eagle's medium were infected with virus-containing allantoic fluid. After 4.5 hrs post infection (p.i.), the medium was removed an [^{14}C]amino acids (50 μCi/ml) in mixture of Eagle's medium with Hanks buffer saline (1:5) were added. The interval of labelling was 4.5 – 5.5 hrs p.i. Afterwards, the cells were scraped off and suspended in STE (10 mmol/l Tris HCl, 100 mmol/l NaCl, 1 mmol/l EDTA, pH 7.4). One fifth of this suspension was pelleted by centrifugation and lysed in sample buffer for polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). The remaining 4/5 of the suspension was tested in RIPA according to Dantas *et al.* (1986) with slight modifications: the *Staphylococcus aureus* A antigen was used instead of protein A-Sepharose.

The infectivity neutralization of original viruses and their MA variants was performed *in vitro* by a routine test based on CPE in MDCK cells. The viruses were diluted in Eagle's medium to obtain an infection dose of 100 $TCID_{50}$ (approximately 70 PFU) and mixed with different amounts of MoAb C179. The final concentrations of the MoAb were 100 μg/ml, 10 μg/ml, and 1 μg/ml. After an incubation at 37°C for 45 mins the virus-MoAb mixtures were serially 10-fold diluted in the same medium supplemented with 0.2% bovine serum albumine and 1 μg/ml of trypsin, and these dilutions were added to MDCK cells in 96-well flat bottom

microtiter plates. After 72 hrs of incubation at 37°C in 5% CO_2 atmosphere CPE was read.

Fig. 1 shows the precipitability of HA of original strains and their MA variants with MoAb C179. HA of Dk/NJ/78 and Dk/NJ/78-MA viruses appeared as two bands: the immature, underglycosylated form of HA (the lower band), and the mature, glycosylated form (the upper band) (Sklyanskaya *et al.*, 1980). MoAb C179 completely precipitated both forms of HA of the original strain Dk/NJ/78. In the case of its MA variant, the MoAb reacted with the lower band only. HA of USSR/77 and USSR/77-MA exhibited just one diffuse band with the mature, glycolysated form of HA in its upper part, and the underglycosylated or partly glucosylated form in its lower part (Sklyanskaya *et al.*, 1980). There was no difference in the precipitability of HA with MoAb C179 between USSR/77 and USSR/77-MA: the MoAb precipitated completely the diffuse band of HA of the original strain HA as well as that of its MA variant.

The results of RIPA indicate that whereas the stem region of the glycosylated HA of Dk/NJ/78-MA did not react with MoAb recognizing conformational epitope in contrast to the original strain, there was no difference in the recognition of the immature, underglycosylated HA of the two variants (Fig. 1).

Table 1. Infectivity neutralization with MoAb C179

Viruses	MoAb C179 concentrations (μg/ml)		
	100	10	1
Dk/NJ/78	≥1.875	1.250	0.625
Dk/NJ/78-MA	≥1.0	0.5	0
USSR/77	≥1.5	≥1.5	1.0
USSR/77-MA	≥1.25	≥1.25	1.0

The results are differences between log values of $TCID_{50}$ of the control and those of the used concentrations of MoAbs.

These results correllate with the data of the neutralization test performed with the same MoAb (Table 1). The original strain Dk/NJ/78 was neutralized efficiently at the two higher concentrations of MoAb C179 (100 μg/ml, 10 μg/ml) and partially at 1 μg/ml, while the neutralization of its MA variant at the same concentrations of MoAb was less efficient; 1 μg/ml of MoAb C179 had no effect on the infectivity of Dk/NJ/78-MA. The neutralization activity of MoAb C179 against USSR/77 did not distinguish the original strain from its MA variant. The MoAb neutralized both viruses at the same concentrations (Table 1).

The data of RIPA and neutralization test with MoAb C179, Dk/NJ/78 and Dk/NJ/70-MA allow to assume that an additional site(s) of glycosylation acquired by HA molecule in the course of adaptation to mice (Lipatov *et al.*, 1995) masks the conformational antigenic epitope in the stem region of HA and brings about a change in the accessibility of this antigenic site to MoAb C179. HA of MA variant of USSR/77 cannot be discerned from that of the original strain in reactions with the MoAb recognizing conformational epitope. The absence of differences between HA of USSR/77 and that of USSR/77-MA in the capacity to be recognized and neutralized with MoAb C179 indicates that the loss of glycosylation sites in the HA_1 subunit of HA in the process of adaptation to mice (Gitelman *et al.*, 1986; Shilov *et al.*, 1994) does not influence the conformation of the stem region and its recognition with MoAb C179.

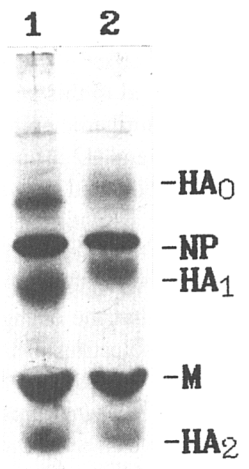

Fig. 2
PAGE of proteins of influenza virus strain Dk/NJ/78 and its MA variant
Virus-containing allantoic fluid was clarified by centrifugation at 15,000 rpm for 20 mins at 4-5°C. Virions were pelleted by centrifugation through 20% (w/v) sucrose cushion at 23,000 rpm for 2 hrs at 4-5°C in rotor SW 27.1 of Beckman ultracentrifuge L5-65. The pellets were lysed in sample buffer and subjected to PAGE (for conditions see Fig. 1). Gels were stained with Coomassie Blue R-350 (Pharmacia). Lane 1: Dk/NJ/78; lane 2: Dk/NJ/78-MA.

To determine the localization of the additional glycosylation site in HA of Dk/NJ/78-MA, the structural proteins of the original strain Dk/NJ/78 and its MA variant were studied PAGE. It revealed clear-cut differences in the mobility of HA_1 subunit between the original strain and its MA variant (Fig. 2). The molecular mass of the MA variant HA_1 subunit was higher than that of the original strain. This result indicates that an additional site(s) of glycosylation

acquired in the course of adaptation to mice and affecting the accessibility of the stem region to MoAb C179 resides in the HA_1 subunit of HA.

The results obtained in this study provide evidence that the conformational antigenic epitope in the stem region of HA conserved in H1 and H2 influenza virus strains (Okuno *et al.*, 1993) does not depend on the host origin of the viruses, although the glycosylation of the HA_1 subunit in the course of adaptation to mice may influence the recognition of this region of HA with MoAbs with conformational activity. It is possible that the changes of HA of Dk/NJ/78-MA are a result of selection in the organism of mice by antibodies similar to MoAb C179. We will attempt to confirm the results of the *in vitro* experiments by *in vivo* studies where the protective effect of MoAb C179 on lethal bronchopneumonia caused by MA variants of influenza viruses belonging to H1 and H2 subtypes will be investigated.

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