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Specific Biochemical Features of Replication of Clinical Influenza Viruses in Human Intestinal Cell Culture

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Abstract—Influenza A viruses isolated from the respiratory tract of patients with influenza were cultured in human intestinal epithelium cells (CACO-2 line). The CACO-2 cells were found to be 100-fold more susceptible to the clinical viruses than MDCK cells and chicken embryos. On passaging in CACO-2 cells, clinical isolates of the subtype H3N2 retained the original "human" phenotype and agglutinated human but not chicken erythrocytes, whereas on passaging in MDCK cells the virus phenotype changed to the "avian" one. On comparison with laboratory strains (grown in chicken embryos or MDCK cells), the clinical viruses were characterized by higher stability of the anti-interferon protein NS1 but had a reduced synthesis of the matrix protein M1, and this could facilitate the virus adaptation and escape of the infected cells from immune attack in the human body. The increased tropism to the human CACO-2 cells correlated with higher adsorption of the clinical viruses on cellular receptors. However, in the CACO-2 and MDCK cells the ratio of sialyl-containing glycoreceptors of the 2-3 and 2-6 type was similar. These observations indicated that not only sialic acid residues were involved in the adsorption and penetration of the clinical viruses into human cells, but also the protein moiety of the cellular receptor itself and/or an additional cellular coreceptor. Thus, clinical influenza viruses are shown to possess a specific mechanism of sorption and entry into human epithelial cells, which is responsible for their higher tropism to human cells and is unlike such a mechanism in canine cells.

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Replication of influenza virus in host cells is known to depend on cellular factors. One such factor is the influenza-activating protease (IAP), which specifically cleaves viral hemagglutinin (HA0) into two subunits, HA1 and HA2, and makes the virus infectious for target cells [1, 2]. The presence of such a protease in the CACO-2 cell culture is responsible for the multicycle replication of the virus. In its absence, only one cycle of virus replication occurs resulting in production of noninfectious virions with the uncleaved protein. To compensate for the lack of the activating protease, many cultures are supplemented with exogenous trypsin, which effectively cleaves HA0 with production of HA1 and HA2 and permits multicycle virus replication [1].

For replication, influenza virus also needs the presence on the cell surface of receptors containing a terminal residue of sialic (N-acetylneuraminic or N-glycolylneuraminic) acid [3]. Two main types of such receptors of influenza virus are known. In the first type of receptors, sialic acid is bound with galactose of a carbohydrate moiety through the α2-3 bond, and in the other type there is the α2-6 bond. Human influenza viruses use the 2-6 type receptors, whereas avian and horse influenza viruses are mainly adsorbed on 2-3 type receptors [4-9]. Notably,

Abbreviations: CACO-2) permanent cell line of human intestinal epithelium; C-virus) virus passed in CACO-2 cells; HA) hemagglutinin; HAA) hemagglutinating activity; MDCK) permanent cell line of canine kidney; MMA) lectin isolated from *Maackia amurensis*; M-virus) virus passed in MDCK cells; NA) neuraminidase; RT-PCR) polymerase chain reaction with reverse transcription; SNA) lectin isolated from *Sambucus nigra*; TMB) tetramethylbenzidine; VERO) permanent cell line of monkey kidney.

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erythrocytes of different animal species are characterized by different ratio of sialic receptors on their surface and, therefore, are selectively agglutinated by human and avian viruses. The viral property of selective agglutination of erythrocytes of different animals is used for identification of the "avian" and "human" viral phenotypes [10-12].

The receptor selectivity underlies the adaptation process of the virus to different hosts. Thus, avian influenza viruses recognizing the 2-3 receptors change their receptor affinity for the 2-6 type on adaptation to humans [9, 10, 13, 14]. On the contrary, human influenza viruses recognizing the 2-6 type receptors during passages in the allantoid cavity of chicken embryos enriched with the 2-3 type receptors change their receptor profile for 2-3 [10, 13]. The change in the receptor specificity of the virus is accompanied by alteration of the receptor site in its hemagglutinin. Positions 226 and 228 are the most significant in the receptor site; they are, respectively, Leu and Ser in human viruses and Gln and Gly in avian and horse viruses [4, 5, 14-18]. The receptor activity of HA is reciprocally coupled with the function of viral neuraminidase (NA). During adaptive changes in the virus, the increase in adsorptive properties of HA was accompanied by increase in the NA properties, and the decrease in the functions of either of them was associated with the decrease in the activity of the other [19].

Variability of the cell reception manifests itself during the isolation of viruses from humans by propagation in chicken embryos and cell cultures. The adaptive variability of influenza virus was first detected by Burnet et al. [20, 21]. They found that influenza viruses were rapidly isolated from patients on passaging in the amniotic cavity of chicken embryos and acquired the ability to multiply in the allantoic cavity of the embryos only after a series of passages in it. The authors referred to these passage variants as "O" (original) and "D" (derived) [21]. The initial O-virus agglutinated human and guinea pig erythrocytes and failed to agglutinate chicken erythrocytes (the "human" phenotype), whereas the D-virus easily agglutinated chicken erythrocytes, along with inferior agglutination of human and guinea pig erythrocytes (the "avian" phenotype). Furthermore, it was established that properties of the O and D variants coincided with phenotypes of the human and avian influenza viruses, and the last terms became more common [10-12]. Viruses from patients with influenza were also isolated in cell cultures, such as BHK-21, LLC-MK, MRC-5, VERO, and MDCK. Passages of the H3N2 subtype viruses in hamster and canine cells (BHK-21 and MDCK, respectively) were usually associated with changes in the receptor properties of the virus [11, 12, 22], whereas on isolation of clinical viruses in monkey cells VERO their receptor phenotype did not change [23]. In some cases the isolated viruses displayed changes in the receptor site region of the HA protein compared to the initial clinical viruses [11, 21, 22], whereas in other cases no mutations were found in the HA protein [23]. Based on these observations, the carbohydrate component of the viral glycoprotein HA was suggested to be involved in the regulation of its receptor specificity [23].

Cell culture of human colon intestinal epithelium (the CACO-2 line) was earlier shown to maintain the multicycle reproduction of influenza viruses because it contained a protease necessary for cleavage of viral HA0 into HA1 and HA2 [24, 25]. We found that the CACO-2 culture had additional advantages because it was more susceptible for isolation of clinical influenza viruses than chicken embryos and the MDCK cells. The clinical viruses passaged in the CACO-2 cells (C-viruses) retained the initial human O-phenotype, while the clinical viruses passaged in the MDCK culture (M-viruses) acquired the D-phenotype. C-Viruses infected the CACO-2 cells 100-fold more effectively than the MDCK cells, whereas M-viruses were polyspecific and similarly infective for both cell cultures. The increased tropism to the human cells correlated with higher adsorption of the clinical viruses on the receptors of the CACO-2 cells. However, the specific sorption of SNA and MMA lectins revealed similar ratios between the sialyl-containing glycoreceptors of 2-3 and 2-6 types in the CACO-2 and MDCK cells. This observation indicated that not only sialic acid residues were involved in the adsorption and entry of the clinical influenza viruses in the human cells, but also the protein moiety of the cellular receptor itself and/or an additional cellular coreceptor that enhanced the viral infection of the cells. Thus, the findings have shown that clinical influenza viruses have a specific mechanism providing their sorption and entry into the human epithelial cells (CACO-2), and this mechanism, first, is different from such of the canine cells (MDCK) and, second, is responsible for their higher tropism to human cells than to cells of other origin.

MATERIALS AND METHODS

Cells and viruses. The MDCK-2 cell culture was from the Philipps University collection (Germany) and the CACO-2 cells were from the European collection (ECACC). These cultures were grown in modified Dulbecco medium (DMEM; Gibco/BRL, Germany) supplemented with 10% fetal calf serum (Gibco/BRL). Specimens of clinical influenza viruses were obtained by taking nasopharyngeal washings from patients with influenza at Moscow Infectious Disease Clinics No. 1 in winter 2003. The washings were performed with sterile saline supplemented with penicillin (100 U/ml), streptomycin (10 μg/ml), and kanamycin (50 μg/ml) in volume of 10 ml per patient. Then the material was precipitated at 4000 rpm at 4°C. The precipitates were suspended in 0.4 ml of DMEM with antibiotics and ultrasonicated for 3 min using a Branson 4500 sonicator. The resulting homogenate was diluted in DMEM with antibiotics,

introduced into the cell culture, and incubated in the presence of CO_2 at 37°C. During the incubation, the emergence of virus was determined by hemagglutination of 1% suspension of human (O(I) blood group), chicken, and guinea pig erythrocytes. On appearance of the hemagglutinating activity, the culture fluid (the first passage) was filtered through a Millex-GV filter (0.22 μm) and used for the further investigations. The isolated in CACO-2 cell culture influenza viruses from different patients were referred to as A/Moscow/235/2003 (isolated February 2003), A/Moscow/328/2003 (February 2003), A/Moscow/343/2003 (March 2003), A/Moscow/346/2003 (March 2003), A/Moscow/450/2003 (January 2003), and A/Moscow/352/03 (January 2003). Nucleotide sequences of the genes *HA*, *NA, M*, and *NS* of these viruses were deposited in GenBank (with accession codes: DQ066936; DQ066937; DQ086157-DQ086161; DQ089634-DQ089639; DQ090706-DQ090710; DQ091199; DQ096580; DQ098261-098269; DQ100422-100424; DQ886602; DQ886603; DQ887750-DQ887752).

Hemagglutinating activity (HAA). HAA was determined by double dilutions of the specimen under study in the volume of 100 μl with 1% suspension of O(I) group human, guinea pig, and chicken erythrocytes prepared in phosphate buffer saline (PBS: 10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.2), 2.7 mM KCl, 137 mM NaCl). The last dilution of the specimen under study with a distinct hemagglutination was taken as the hemagglutinating titer.

Reverse transcription reaction-PCR (RT-PCR). Viral RNA was isolated from clinical homogenates and culture fluid of the CACO-2 and MDCK cells using a High Purification Kit (Roche, Switzerland), which provided for a stage of treatment of the preparation with type I DNase to reduce a cellular DNA contamination. From the resulting RNA-template, the DNA-product was synthesized using a One Step RT-PCR Kit (Qiagen, Germany) and primers specific for the 3′- and 5′-terminal regions of the viral genes *HA* and *NA* [26], and then the secondary PCR was performed using shifted primers. The primers used were as follows:

HA–1/Fo, 5′-A GGG AGC AAA AGC AGG GG-3′;

AiPR–HA1012/Fo, 5′-CAC CCT GAG GAT GGC AAC AGG-3′;

HA1370/fo5, 5′-GCC CTG GAG AAC CAA CAT AC-3′;

HA1,3/529/re1, 5′-AGT CAC GTT CAG CGC TGG ATA T-3′;

HA1,3/1021/re2, 5′-CGG AAC ATT CCG CAT CCC TGT-3′;

HA1,3/1477/re3, 5′-AGG CAT TGT CAC ATT TGT GGT A-3′;

NA–Klon-EarI/fo, 5′-ATA TCT CTT CGG CCA GCA AAA GCA GG GTG-3′;

NA–Klon–EarI/re, 5′-ATA TCT CTT CTA TTA GTA GAA ACA AGG GTG TTTT-3′;

N2spe1150/re, 5′-CCA GCC TTC AAT GAC TTT G-3′;

N1spe1150/re, 5′-CCA CCC ATT TGG ATC CCA A-3′.

Method of immune foci in cell culture. A monolayer of the CACO-2 or MDCK cells in 24-well plates was infected at 37°C for 1 h with viruses in different dilutions, and upon the infecting the cell monolayer was covered with 0.8 ml of 1% agarose in Dulbecco medium supplemented with 0.05% BSA, penicillin (100 U/ml), and streptomycin (25 μg/ml), and also trypsin (0.3 μg/ml) in the case of MDCK. After 45-60 h, the cells were fixed for 4 h in 2% *p*-formaldehyde in PBS and permeabilized in 0.2% solution of nonionic detergent NP-40 for 15 min. Then the monolayer was incubated for 1 h at 20°C with monoclonal antibodies to the influenza protein NP (clones A1 or A3, collection of the Center for Disease Control, USA) or to type H3 and H1 protein HA (collection of Philipps University, Germany) and then with the anti-species peroxidase conjugate. The immune complexes were stained with water-insoluble TMB substrate True Blue (KPL, USA).

Sorption of specific lectins on cellular receptors. To determine sialyl-containing receptors of 2-3 and 2-6 type on the MDCK and CACO-2 cells, specific lectins were used isolated from *Sambucus nigra* (SNA) (Calbiochem, USA) and *Maackia amurensis* (MAA) (Sigma, USA) which acted as specific ligands for these receptors, respectively [27, 28]. Solutions of these lectins (2 mg/ml) were mildly labeled with a low concentration (0.2 mg/ml) of NHS-biotin (Calbiochem) for 5 min, with a subsequent inactivation of free NHS-biotin by excess of glycine. Different cells grown in 96-well plates were washed in PBS supplemented with $CaCl_2$ and $MgCl_2$ in the final concentrations of 1.2 mM (PBS++) and then incubated for 50 min on ice with two-fold dilutions of biotinylated lectins prepared in 20 mM Tris-HCl buffer (pH 7.7) supplemented with 0.15 M NaCl, 0.05% BSA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, starting from 3 and 25 μg/ml for SNA and MAA, respectively. Then the cells were washed in PBS++, fixed in 2% *p*-formaldehyde for 30 min, and incubated with streptavidin–peroxidase conjugate (Amersham-Biotech, England), with a subsequent staining of the complexes with water-insoluble TMB-substrate. The stained preparations were examined using a light microscope. The relative content of lectin-positive cells was determined in the previous to the last dilution with a still detectable staining.

Assessment of cellular adsorption of virus. The viruses A/Moscow/328/2003, A/Moscow/343/2003, and

A/Moscow/346/2003 were grown for 24-48 h in the CACO-2 or MDCK culture in DMEM medium without serum. The culture fluid containing 2^3-2^5 hemagglutinating units (HAU) per ml was cleared at 8000*g* for 20 min and incubated with NHS-biotin (Pierce, USA) (0.3 mg/ml) for 20 min. The biotinylation was stopped by addition of Tris-HCl (pH 7.5) to the final concentration of 50 mM. The biotinylated virus was precipitated through 4 ml of 20% sucrose in PBS using an SW-41 rotor (Spinco model L7) at 23,000 rpm for 2.5 h and resuspended in PB to the final virus concentration of 2^8 HAU/ml. The resulting virus was incubated with the MDCK or CACO-2 cells. These cells were grown in 96-well plates, washed in PBS containing 10 mM $CaCl_2$ and $MgCl_2$ (PBS++), and incubated for 60 min on ice with double dilutions of the biotinylated virus in PB++. Upon the adsorption, the cells were washed in PBS++, fixed with 2% *p*-formaldehyde for 30 min, incubated with streptavidin–peroxidase conjugate (Amersham-Biotech), and the complexes were stained with water-insoluble TMB substrate. The stained preparations were photographed at 250-fold magnification using a light microscope.

Electrophoresis in polyacrylamide gel and identification of proteins by western blotting. SDS-PAGE of polypeptides was performed in a mini-apparatus. Before the analysis, the specimens were dissolved in buffer containing 1% SDS, 10 mM dithiothreitol (DTT), and 10% glycerol. Upon the electrophoresis, the polypeptides were semi-dry transferred onto a Protran membrane (Schleicher & Schuell, Germany). The membrane was incubated with antiviral antibodies in PBS supplemented with 0.5% BSA for 1.5 h at 20°C, and the immune complexes were identified using an antispecies peroxidase conjugate (Dako, Denmark) by enhanced chemiluminescence (ECL) with ECL-supersubstrate (Pierce).

RESULTS

In the first part of the work, the susceptibility of the CACO-2 and MDCK cell cultures to clinical viruses was compared. The nasopharyngeal washings from patients with influenza were introduced into the cell culture, and during the incubation the presence of the viral hemagglutinating activity was tested using human (O(I) blood group), guinea pig, and chicken erythrocytes. Usually HAA was recorded on the 5-8 day (first passage) after the introduction of patients' specimens into the cell culture. We succeeded in the isolation of virus from eight of sixteen patients in the CACO-2 culture. Seven of these eight isolated viruses agglutinated human erythrocytes and did not agglutinate chicken erythrocytes ("human" phenotype), and one isolate (strain A/Moscow/450/03) agglutinated chicken erythrocytes at considerably higher dilutions than human erythrocytes ("avian" phenotype of agglutination). Attempts of parallel isolation of virus in the MDCK cell culture with addition of exogenous trypsin were less successful, and the A/Moscow/450/03 virus was isolated from only one patient.

After the HAA had been recorded in the first passage, the subsequent passages were performed on the CACO-2 and MDCK cultures with the multiplicity of infection (MOI) of about one plaque-forming unit (PFU) per 100 cells. The passage variants obtained on the CACO-2 and MDCK cells were referred to as C- and M-viruses, respectively. On passaging in the CACO-2 cells, the viral hemagglutinating activity was revealed only with human erythrocytes and was not recorded with chicken erythrocytes (Fig. 1). Such a profile of the erythrocyte agglutination was stable and retained for at least 20 passages. According to previous works, such an agglutination profile is specific for human influenza viruses possessing affinity for sialic receptors with the 2-6 type bond [12]. The agglutination profile considerably changed during the passaging of the clinical isolates in the MDCK cells. Even after three to five passages in the MDCK cells, the virus began to agglutinate chicken erythrocytes at higher titers than human erythrocytes, i.e., the virus acquired the "avian" phenotype. Based on these observations, it was concluded that the passaging in the CACO-2 and MDCK cell cultures was associated with selection of viruses with different hemagglutinating properties.

Then the ability of the isolated viruses to multiply in the CACO-2 and MDCK cell cultures was studied, and the antigenic type of hemagglutinin and neuraminidase was identified. This was performed by three methods: western blotting with the antibodies specific to H1 and H3 type hemagglutinin, immune foci in the cell culture, and PCR with primers specific for the viral genes. Seven of the eight isolated strains were found to have the H3 type hemagglutinin, and strain A/Moscow/450/03 had H1 type hemagglutinin (Fig. 2, a and b). The immune staining of the foci in the CACO-2 culture revealed a similar staining of the viral foci with the antibodies to H3 in seven isolates and with anti-H1 antibodies in one isolate (not shown). In addition to the HA type, the type of viral neuraminidase was determined by western blotting using antibodies to neuraminidase N2 (collection of the Center for Disease Control, USA). This type of neuraminidase was found in all isolated viruses with the H3 genotype (not shown). This finding was also confirmed by the RT-PCR with the primers specific for the *N1* and *N2* genes (Fig. 2c). Thus, the isolated influenza viruses were concluded to belong to the H3N2 type (seven isolates) and the H1N1 type (one isolate).

Then the synthesis of individual viral proteins was compared in the CACO-2 and MDCK cell lines infected by C- and M-viruses. Synthesis was identified by western blotting with antibodies specific for the viral proteins HA, NP, M1, and NS1. In CACO-2 and MDCK cells infected with clinical isolates of influenza viruses, the synthesis of the viral protein M1 was markedly lower than the NP pro-

tein synthesis, as compared to the laboratory virus A/Aichi/2/68 (Fig. 2d). In the MDCK line cells, protein NS1 of clinical viruses was degraded with production of low-molecular-weight products (indicated by the asterisks in Fig. 2e). The protein M1 deficiency found in the cells infected with clinical influenza viruses could lead to limitation of their reproduction. The mechanism of decreased synthesis of the viral protein M1 of clinical viruses is still unclear, but a decreased secondary transcription is probable of the late genes, including the gene *M*, in clinical isolates in the CACO-2 and MDCK line cells.

Then the replication of clinical viruses in the CACO-2 and MDCK cells was studied by the method of immune foci. The CACO-2 and MDCK cell cultures were infected by viruses passaged in the CACO-2 cells (C-virus) and MDCK cells (M-virus) and incubated under agarose cover for three days, while observing the size of viral foci. First, the clinical isolates were found to differ in the ability to infect human and canine cells. Thus, the C-viruses were 10^2-10^4 more infective for the CACO-2 cells than for the MDCK cells, whereas the M-viruses did not display such a difference and had a similar tropism to both cell cultures (Fig. 3). Second, the isolates of clinical viruses were pronouncedly heterogeneous in the ability to replicate in the cell cultures and produced foci of different size. This heterogeneity was more prominent in the M-virus isolates (Fig. 3). Third, the laboratory viruses A/Aichi/68 (H3N2) and A/PR/8/34 (H1N1), which had undergone many passages in chicken embryos, lacked the cellular selectivity and effectively replicated in both cell cultures producing large foci of similar size.

Then the virus adsorption on receptors of the CACO-2 and MDCK cell lines was studied. The C- and M-viruses were biotinylated and incubated with the cells

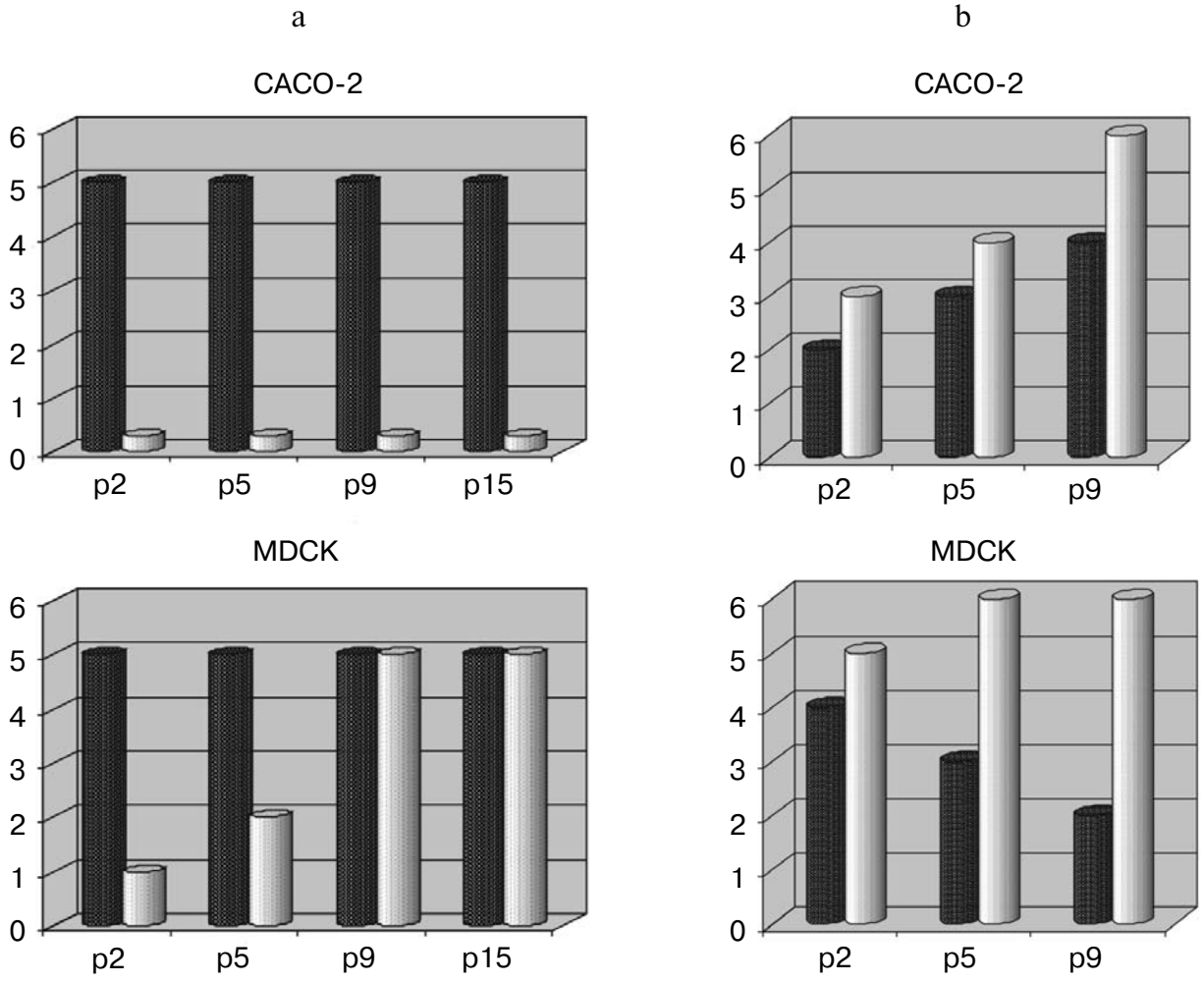

Fig. 1. Changes in hemagglutinating profile of clinical viruses A/Moscow/328/03 (H3N2) (a) and A/Moscow/450/2003 (H1N1) (b) passaged in CACO-2 and MDCK cultures. The patients' nasopharyngeal washings were passaged in the CACO-2 and MDCK cultures (MOI ~ 0.01), and the hemagglutinating titer was determined in two-fold dilutions of the culture fluid with 1% suspension of chicken embryo and human O(I) group erythrocytes (light and dark columns, respectively). Ordinate, $\log_2$ of the value inverse to the last dilution of the culture fluid with the recorded agglutination of erythrocytes; abscissa, the passage number in the cell culture.

at the temperature of about 0°C to prevent internalization of the adsorbed virus into the cells. The virus adsorption was assessed by biotin-specific staining of the cells. The viruses that were passaged in the CACO-2 cells were more effectively adsorbed on the CACO-2 than on the MDCK cells, whereas the M-viruses were adsorbed similarly on the CACO-2 and MDCK cells (Fig. 4). These finding indicated that clinical viruses passaged in the CACO-2 cells retained higher affinity for the human cellular receptors, while their passaging in the MDCK cells abolished this selectivity. The selective sorption of the C-virus on the CACO-2 cells correlated with its higher infectivity and tropism to these cells.

Differences in adsorption of influenza viruses are known to be associated with the different ratio between 2-3 and 2-6 receptors on the cells [14]. We determined contents of the receptors in the CACO-2 and MDCK cells using the selective binding of the SNA and MAA lectins, which are specific for the sialyl-containing receptors of the 2-3 and 2-6 type, respectively. Monolayers of the growing CACO-2 and MDCK cells were incubated with prebiotinylated lectins, the lectins bound in the cell monolayer were identified by biotin-specific staining with peroxidase–streptavidin conjugate and TMB, and the quantity of stained (positive) cells was determined. The two cell cultures, CACO-2 and

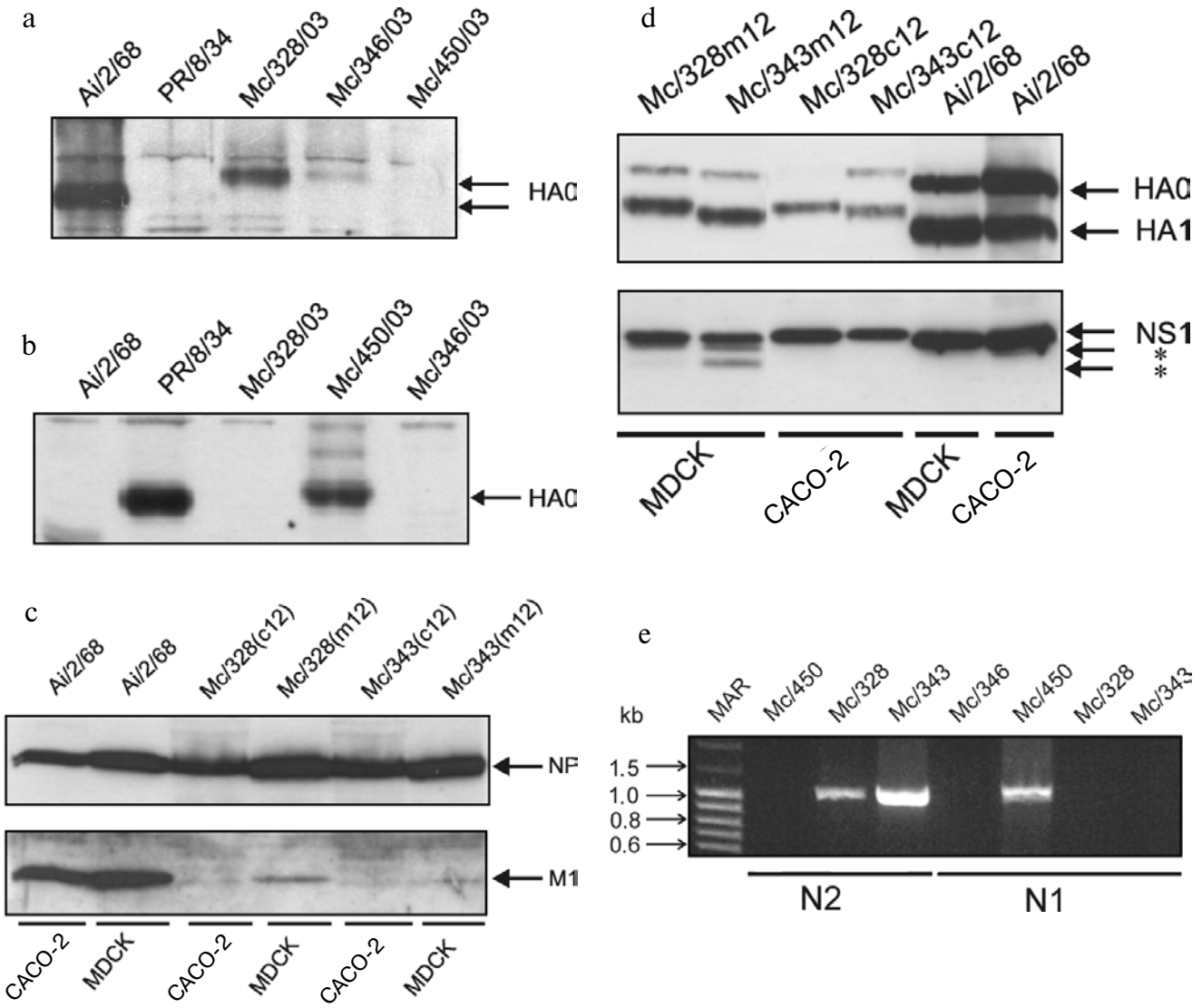

Fig. 2. Typing of hemagglutinin of clinical viruses by western blotting and PCR. The CACO-2 or MDCK cell culture was infected with viruses after 12 or 13 passages in CACO-2 or MDCK cells: A/Moscow/328/03 (Mc/328/03), A/Moscow/343/03 (Mc/343/03), A/Moscow/346/03 (Mc/346/03), A/Moscow/450/03 (Mc/450/03), with MOI 1 PFU/cell. The viruses A/Aichi/2/68 (H3N2) (Ai/2/68) and A/PR/8/34 (H1N1) (Pr/8/34) were passaged in chicken embryos. At 18 h after the infection, the cellular proteins were analyzed by western blotting with specific anti-H3 (a), -H1 (b), -NP and -M1 (c), -HA and -NS1 (d) antibodies and with the antispecies peroxidase conjugate by enhanced chemiluminescence. RNA from the infected cells was analyzed by RT-PCR with primers specific for the *NA* gene of the N1 and N2 subtypes (e).

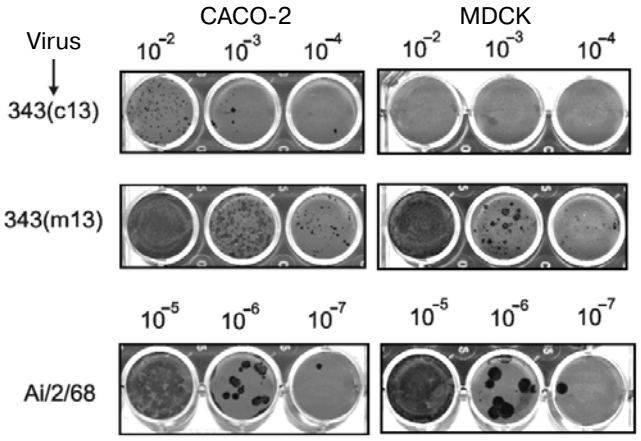

Fig. 3. Production of foci by C- and M-variants of A/Moscow/343/2003 virus in the CACO-2 and MDCK cell cultures. Tenfold dilutions of the virus A/Moscow/343/2003 (view from above), which underwent 13 passages in the CACO-2 (c13) and MDCK (m13) cells, were inoculated into the CACO-2 and MDCK cultures and incubated under an agarose layer supplemented with trypsin (in the case of MDCK) for three days. The viral foci were stained with antibodies against the viral protein NP using the peroxidase conjugate and water-insoluble TMB. Ai/2/68 is the control virus A/Aichi/2/68 (H3N2) grown in chicken embryos.

MDCK, effectively bound both lectins, and this suggested high content of both 2-3 and 2-6 type receptors (Table 1). The ratio of the cells enriched with the 2-3 and 2-6 receptors was similar in the CACO-2 and MDCK cultures. Fractions of the cells positive by the 2-6 and 2-3 receptors were 62 and 57% of the total cell population in the CACO-2 culture and 60 and 54% in the MDCK culture, respectively. Thus, the two cultures were rich in both type receptors, and it provided for the high adsorption of viruses with both the "avian" and "human" phenotype. These data allowed us to neglect the trivial explanation of the higher affinity of the C-virus for the CACO-2 cells than for the MDCK by the higher content of the 2-6 receptors.

The CACO-2 and MDCK had similar contents of the 2-6 type sialyl receptors; therefore, it was suggested that the difference in the adsorption of the C- and M-viruses on the CACO-2 and MDCK cells should be caused by specific features of the surface viral proteins HA and NA. These specific features could belong to both the protein and carbohydrate moieties of the protein molecules. To elucidate the role of the carbohydrate moiety and secondary cell-specific modifications (glycosyla-

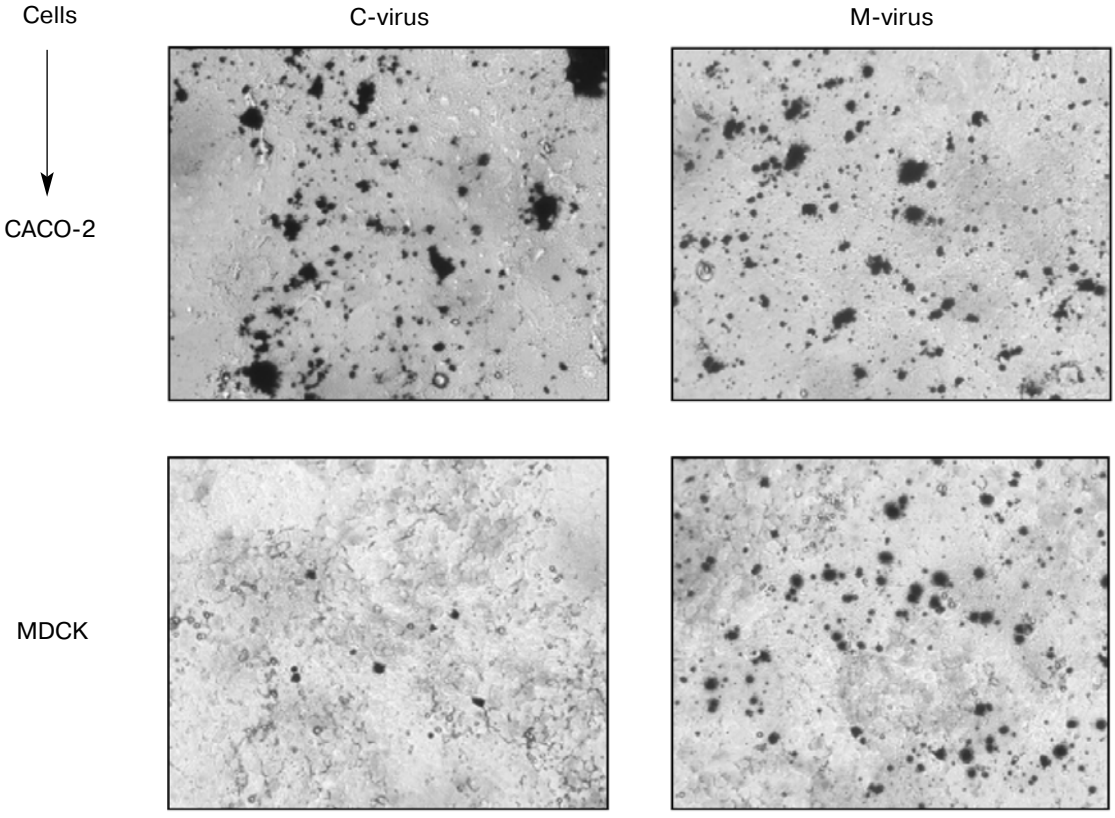

Fig. 4. Adsorption of the C- and M-virus isolates in CACO-2 and MDCK cells cultures. Isolates of A/Moscow/343/03 virus passaged 12 times in CACO-2 and MDCK cultures (C- and M-virus, respectively) were labeled with biotin. Preparations of the biotinylated C- and M-viruses were equalized by the hemagglutinating activity and incubated with the CACO-2 and MDCK cells (64 HAU/cell). The quantity of the virus adsorbed on the cells was identified using the peroxidase–streptavidin conjugate with subsequent staining by TMB. The resulting preparations were photographed with a digital microscope (×250).

Table 1. Binding of SNA and MAA lectins with CACO-2 and MDCK cells

Cell line	Adsorption of lectins, %*	
	SNA (2–3)	MAA (2–6)
CACO-2	57.0 ± 22.1	62.4 ± 25.0
MDCK	54.0 ± 23.8	60.3 ± 26.2

* The biotinylated SNA and MAA lectins specific for sialyl receptors with 2-3 and 2-6 terminal bonds, respectively, were incubated with CACO-2 and MDCK cells. The lectins adsorbed on the cells were identified using peroxidase–streptavidin conjugate and subsequent staining with TMB. The resulting cell preparations were photographed with a digital microscope (×100). The quantity of cells with the adsorbed lectins was evaluated by the area occupied by the stained cells (%) in 30 random fields of vision (mean value ± standard deviation).

tion, sulfatation, acylation, etc.) of the HA and NA proteins in the adsorption of the viruses, the C-virus was cross-replicated in the MDCK and the M-virus was cross-replicated in the CACO-2 cells. Host change is known to cause a change in the carbohydrate moiety of viral proteins, because the glycosylation type depends on the host cell [17, 29]. After the only cross-replication of the virus its hemagglutinating profile with different erythrocytes and the infectivity of its virus progeny for the CACO-2 and MDCK cells were studied, and amounts of the newly synthesized virus in the cell medium and on the infected cells were also evaluated. First, in both cases the host change was not accompanied by considerable changes in the hemagglutinating profile of the virus (Table 2). The virus passaged in the CACO-2 cell culture and possessing the "human" phenotype retained its profile after the single replication in the MDCK cells and agglutinated only human erythrocytes. The M-virus also did not change its "avian" phenotype after the replication in the human cell culture CACO-2 and agglutinated both types of erythrocytes. Thus, the change in the type of glycosylation and other secondary modifications of the CACO-2-specific ("human") type by the MDCK-specific ("canine") type and *vice versa* in the viral proteins HA and NA did not change the adsorption of the virus. Second, in the cell systems studied, the virus ratio on the cells and in the extracellular medium was different. The virus content was high on the MDCK cells infected by the C-virus and it was associated with formation of small-size foci (Table 2). These two observations can be easily ascribed to the weak elution by neuraminidase of the M-virus from the CACO-2 cells at the stage of viral budding and its insufficient propagation onto the neighboring cells.

DISCUSSION

In the present work, biochemical features of clinical influenza virus were studied during the replication in the culture of human intestinal epithelium cells (CACO-2). These culture cells share properties of enterocytes and features of differentiated epithelium, such as secretory activity and formation of secretory domes, presence of surface microvilli and microvilliferous border, expression of the microvilliferous layer enzymes (sucrase, alkaline phosphatase, etc.) [7, 30]. The CACO-2 cell line is virtually the first finite line of human cells that can provide for

Table 2. Characteristics of the virus progeny in the MDCK and CACO-2 cells infected by the C- and M-viral isolates

Infecting virus	Target cells	HA titer*		Virus content, %*		Foci**
		human	chicken	cells	culture fluid	
A/Moscow/328/03 (C_{13})	MDCK	2^7	$<2^3$	40	60	M
	CACO-2	2^7	$<2^3$	43	57	M
A/Moscow/328/03 (M_{13})	MDCK	2^7	2^7	3	97	M
	CACO-2	2^7	2^6	95	5	S/unit cells
Victoria/75	MDCK	2^8	2^8	4	96	L
	CACO-2	2^8	2^8	3	97	L

* The cell cultures were infected by C- and M-variants of the A/Moscow/328/03 virus that underwent 13 passages (MOI ~ 1) or by the laboratory virus A/Victoria/75 (H3N2) grown in chicken embryos, and 37 h after the infection, the culture fluid and the cellular precipitate (cells) were collected. In these samples, the hemagglutination titer was determined with 1% human and chicken erythrocytes and the virus quantity was evaluated by titration on the CACO-2 cells using the method of immune foci. Considering the obtained values of virus titer and initial volume of the samples, the virus contents (in %) were determined in the cells and culture fluid.

** Virus foci in the CACO-2 and MDCK cells were determined in the cell monolayer under the agarose cover containing trypsin (in the case of MDCK). The virus foci were stained three days after infection and analyzed using a light microscope (×60). By the content of the virus-positive cells in the focus, the foci were characterized as large (L), middle (M), and small (S) containing 250 and more, 100-150, and 1-10 cells per focus, respectively. Unit cells – only separate cells were stained.

a steady multicycle replication of influenza viruses [24, 25]. The human liver cell line Hep-2G was recently recorded to be also susceptible to clinical viruses of human influenza [31]. The previously studied cultures of human cells (HeLa, A-549, the 293 line, etc.), first, displayed deficiencies during the virion assemblage stages, which prevented preparation of considerable virus titers, and, second, had no virus-activating protease that prevented the maintaining of multicycle replication of influenza viruses [32, 33]. The high ability of the epithelial CACO-2 cells to maintain the viral reproduction has allowed us to propose an important hypothesis that the human intestinal epithelium is susceptible to influenza virus and can be involved in the influenza pathogenesis in the human body, inducing the intestinal syndrome [25]. Moreover, the ability of the CACO-2 cells to retain the original phenotypic features of clinical viruses allowed us not only to successfully isolate these viruses from patients, but also to study their biochemical properties. The study on the isolated clinical viruses indicated the homogeneity of the human virus subtype H3N2 population: all isolated strains manifested the "human" type hemagglutination, and this is consistent with data of other authors [10-13, 23, 34]. On the contrary, the human virus population of the subtype H1N1 was heterogeneous, and the three possible phenotypes – "human", "avian", and "mixed" – circulated in humans [35]. The strain A/Moscow/450/03 (H1N1) isolated by us in the CACO-2 culture was identified as an "avian" influenza virus. The population heterogeneity of the H1 viruses could be caused by the less strong regulation of reception of the H1 type viral HA protein on the receptors of various target cells, in comparison to the reception of the virus subtype H3.

The CACO-2 culture of human cells displayed an increased susceptibility to homologous isolates passaged in this culture, than to heterologous variants passaged in the culture of canine cells. Such a homologous tropism allowed us to isolate influenza viruses from the patients in the CACO-2 line of human cells. The regulatory mechanism of this homologous tropism is still unclear. In particular, the increased susceptibility of the CACO-2 cells to homologous passages (by the C-virus) was associated with higher adsorption and infectivity of the virus on these cells than on heterologous cells of the MDCK line. However, both the increased adsorption and infectivity were not associated with the quantitative difference in the contents of the main viral sialyl-containing cellular receptors of the 2-6 or 2-3 type, because this parameter was virtually the same in the MDCK and CACO-2 cells. Consequently, the detected homologous tropism of the virus suggested that something other than terminal residues of sialic acid in the cellular receptor should be involved in the virus reception on target cells, but also the whole transmembrane receptor complex with its cell-specific environment. Such a receptor complex could be produced from oligosaccharide chains and protein skeleton of the receptor itself and additional cellular coreceptor(s), as occurs in the human immunodeficiency virus (HIV) [36] and reoviruses [37]. In this context, it was suggested that the receptor complex of the CACO-2 line cells, including the hypothetical coreceptor apparatus, should be more adequate than in the MDCK line cells for adsorption and internalization of clinical human influenza viruses of the H3N2 subtype and should control their homologous tropism by this mechanism. In *in vivo* works on desialylated MDCK cells [38] and hamster cells (the CHO line) genetically deficient in protein glycosylation [39], and also in *in vitro* works with synthetic oligosaccharide compounds [40], it was reported that not only the terminal residues of sialic acid were involved in the virus adsorption and penetration but also other components of the cellular receptors. Based on these findings, it was also supposed that either another alternative receptor and sialic acid-independent pathway of the virus penetration [38], or a hypothetical cellular coreceptor should act at the stage of virus internalization into the target cell [39]. The nature of the other cellular receptor and/or coreceptor is not yet established for influenza virus.

In addition to the adequate cellular receptor complex, the virus penetration is also determined by the cell-dependent structural modifications of the virus itself due to glycosylation, sulfatation, and acylation of hemagglutinin and neuraminidase. In particular, it has been reported that the carbohydrate moiety of the viral protein HA and its acylation are involved in the regulation of the virus adsorption and penetration into target cells [21, 41]. On comparison of properties of the subtype H3N2 influenza viruses passaged in the canine cells MDCK and monkey cells VERO, the host change and the associated change in the HA type glycosylation were found to influence the ability of the virus to agglutinate erythrocytes of different animals. But contrastingly, in our work the cross-replication of the C- and M-viruses in heterologous cells of the MDCK and CACO-2 lines, respectively, affecting the cell-dependent modifications (glycosylation, acylation, etc.) of viral HA and NA did not change the hemagglutinating profile of the virus strains. This contradiction of the data can be caused by the degree of the cell-dependent differences in glycosylation/acylation of the viral proteins between the pairs VERO and MDCK in work [21] and CACO-2/MDCK in our study. Thus, our findings have shown that in some cases the cell specificity of glycosylation of viral proteins can be insufficiently pronounced to markedly influence the hemagglutinating and receptor properties of the virus.

Apart from the HA protein, viral neuraminidase can also determine the enhanced tropism of clinical viruses to the CACO-2 line cells. In cooperation with the viral HA, neuraminidase is reported to be involved in the virus interaction with cellular receptors [19] and antiviral sialyl-containing inhibitors [42, 43], and also at the stage of

the virus penetration into the target cells [44]. Our finding of a poor elution (realized by viral neuraminidase) of the C-viruses on the MDCK cells (Table 2) are in agreement with the hypothetical involvement of viral NA in the recognition and interaction of the virus with the cellular receptors and possible alteration of this function in the case of host change. It may be that, as in the case of viral glycoprotein HA, the function of NA can be also influenced by the cell-dependent type of its glycosylation, especially the presence and structure of oligosaccharide chains in the active site region. Consequently, as discriminated from viral HA, the change in the cellular modification of NA on the host change CACO-2→MDCK could affect its biochemical features and affinity for the cell receptor complex. This hypothesis is now investigated by us in experiments with artificial recombinant NA mutants.

An interesting regularity was revealed by studies on the synthesis of clinical virus proteins in the infected cells. In the CACO-2 cells infected by clinical isolates of influenza virus, the matrix protein M1 level was lower than in the laboratory viruses adapted to chicken embryos. It seemed paradoxical that clinical viruses could easily replicate in the CACO-2 cells and had a low level of M1, the key protein in the virion assemblage. The M1 deficiency resulted in a considerable decrease in the replication of laboratory influenza viruses [45], and this seemed reasonable, because the selection of the laboratory influenza viruses passaged in MDCK and chicken embryos was performed mainly on the high reproducibility of the virus. It seems that clinical viruses have the minimal synthesis of the M1 providing for a sufficient level of virus reproduction in the human respiratory tract. The cell culture CACO-2 is likely to imitate the situation occurring in the human respiratory epithelium. This property of clinical viruses seems to ensure their advantage for replication in the human body, because it reduces the immune attack of the infected cells by cytotoxic lymphocytes and antibodies that have the M1 protein as a target.

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