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The efficacy of antiviral preparations *in vitro* on the reproduction of influenza virus strains A/H5N1, which caused an epizootic among domesticated birds in summer 2005

The commercial drugs rimantadine, amantadine, ribavirin and arbidol are effective in suppressing in *in vitro* reproduction of highly pathogenic avian influenza A/H5N1 viruses. This study was done on porcine embryo kidney (SPEV) cells and the highly pathogenic A/Duck/Novosibirsk/ 56/05(H5N1) strain from an infected domestic duck (anas platyrhynchos domesticus) in the Lake Chany area, Novosibirsk region.

In mid-July 2005, in western Siberia, there was an epizootic outbreak with high mortality levels among domesticated bird populations, caused by HPAI (highly pathogenic avian influenza) A/H5N1. Within a one-month period, the epizootic had encompassed the Novosibirsk, Omsk, Tyumen, Kurgansk, Chelyabinsk and Altai regions.

Specialists in infectious disease and virology at the Russian Academy of Medical Science collected field material from the epicenter of the epizootic (the area around Lake Chany known as Barabinsk steppe). Strains of the highly pathogenic virus group A/H5N1 were acquired from domestic and wild birds and established in porcine embryo kidney (SPEV) cells and dog embryo (MDCK) cells. Six strains were added to the Russian government's catalog of viruses, corresponding to GenBank-based nucleotide sequences DQ190857, DQ190858, DQ190859, DQ190860, DQ190861 and DQ190862. The amino acid sequences at the point of proteolytic action on hemagglutinin suggested that these strains belong to the HPAI category [4].

Comparative analysis of the nucleotide sequences of all genes for the western Siberian strains HPAI A/H5N1 with their analogous GenBank sequences allowed us to establish a significant proximity to the HPAI A/H5N1 strains which were isolated at the time of the outbreak of illness in mountain geese (*Eulabeia indica*) at Tsinghai Lake (in a western Chinese province) in the spring of the same year.

The above-described epizootic events show that humans are just one step removed from an influenza pandemic – the virus only has to cross the interspecies barrier by reassorting with strains which can circulate in the human population [7-9, 12]. The western Siberian strains HPAI A/H5N1 (2005) are possible precursors to a pandemic reassorting, thus it is critical to research their sensitivity to commercially available chemical antiviral products.

The goal of the following work was to study the effectiveness of amantadine, rimantadine, virasol and arbidol against reproduction of the western Siberian strains HPAI A/H5N1 (2005) in intermixed cell line models.

Materials and Methods

Cell Cultures. Cultures of interwoven porcine embryonic kidney cells (SPEV) were used. The cells were provided by the D. I. Ivanovsky Virology Department of the Russian Academy of Medical Science.

Virus. In the experiments, we used highly pathogenic strain A/Duck/Novosibirsk/56/05, isolated in the summer of 2005 from infected domestic ducks in the Lake Chany area (Zdvinsk region of Novosibirsk oblast) and included in the government listing of viruses (No. 2371).

The experiments were done on plastic 24-well plates (Costar, USA). We used 199 medium and MEM with the addition of 5% fetal calf serum (Gibco, USA), L-glutamin (10 mcM) and antibiotics. All of the virus-containing samples also contained trypsin (Sigma, USA) in the concentration 2 mcg/ml.

Preparations. As antiviral agents, we used: (a) rimantadine (Adamantan, Russia); (b) amantadine (Oleinpharm, Latvia); (c) ribavirin (ICN Pharmaceutical, USA); (d) arbidol (Masterlek, Russia).

Evaluation of antiviral efficacy was done on the basis of standard accepted methods: by determination of influenza A viral titers, viral-specific hemagglutinin titers, the level of viral antigen expression lowering based on immunoenzyme assay (IEA), and the ability of the preparations to inhibit the development of virus-inducing cytopathogenic action (CPA) [1, 2].

The plates containing the experimental and control samples were incubated in a 5% CO_2 atmosphere at 37 degrees Celsius for 72 hours, at which time the antiviral effect of the preparations was determined. For the study of antiviral activity according to immunoenzyme assay, the tests were completed within 20 hours after the cells were infected.

In no case where the preparations were added to non-infected cells (in concentrations up to 45 mcg/ml), were observed any cytotoxic changes in the cell monolayers during the 72-hour period.

Results and discussion

Table 1: The effect of ribavirin on the reproduction of highly pathogenic strain A/Duck/Novosibirsk/56/05 (H5N1) of the influenza virus in SPEV cell culture

Dose of drug, mcg/ml	Infection titer (lg $TCID_{50}$/ml)
0	**6.5**
1.25	**5.40**
2.50	**4.90**
5.0	**3.85**
10.0	**2.25**

Notes on Tables 1 and 2: Results were measured 72 hours after cell infection; amount of infection was 0.1 TCID50/ml; results are the average of three identical experiments.

The results of the action of rimantadine and ribavirin on the reproduction of influenza virus strain A/Duck/Novosibirsk/56/05 (HPAI/H5N1) in SPEV cell cultures is shown in Tables 1 and 2. The action of these preparations on reproduction was shown mostly by the titers of the Group A virus. The results show that the lowering of the

infection titer of virus Group A in the 72-hour period following cell infection is related to the dosage strength.

Table 2: The effect of rimantadine on the reproduction of highly pathogenic strain A/Duck/Novosibirsk/56/05 (H5N1) of the influenza virus in SPEV cell culture

Dose of drug, mcg/ml	Infection titer (lg $TCID_{50}$/ml)
0	**6.5**
0.75	**5.80**
1.25	**5.30**
2.50	**4.75**
5.0	**4.10**

We studied the effects of rimantadine, amantadine, ribavirin and arbidol on viral reproduction (Table 3), which was shown by two markers – lowering of the hemagglutinin titer in liquid culture (MIC-I) and the ability to partially suppress virus-inducing development (MIC-II). From Table 3 it follows that a measure of MIC-I (complete suppression of hemagglutinin titer) was reached with the use of any of the drugs. MIC-II (prevention of the development of CPA by 50% in comparison with the control) was also reached with these preparations within the designated concentrations (see Table 3). It is important to note that all the drugs (amantadine, rimantadine, ribavirin and arbidol) show a selective antiviral effect, since the MIC-I and MIC-II results were achieved with drug concentrations much smaller than those required to produce any cytotoxic changes in the uninfected cell monolayers.

Table 3: The effect of antiviral preparations on the influenza virus *in vitro,* caused by Group A/Duck/Novosibirsk/56/05 (H5N1), in SPEV cell culture

Preparation	MIC-I, mcg/ml	MIC-II, mcg/ml
rimantadine	**1.25**	**1.25**
amantadine	**1.50**	**2.0**
ribavirin	**2.50**	**2.50**
arbidol	**7.0**	**6.50**

Notes: Results were obtained 72 hours after infection; MIC = minimal inhibitory concentration; MIC-I = the first concentration which completely inhibits the formation of viral hemagglutinins in the culture medium; MIC-II = the concentration which inhibits virus-specific CPA by 50% in comparison with the infected control cells. The infected control cells have an accepted CPA value of 100%.

The results for amantadine, rimantadine and ribavirin are congruent with existing literature from the 1970's and 1980's. The results for the efficacy of arbidol, shown in Table 3, agree with the data of Ye. I. Burtseva, who studied the antiinfluenza effect of this preparation by the spot method in MDCK cell culture.

It was also shown (see figure below) that rimantadine, arbidol and ribavirin suppress reproduction of highly pathogenic strain A/Duck/Novosibirsk/56/05 (HPAI/H5N1) within 20 hours of cell infection, that is, they suppress viral antigen expression, as determined by immunoenzyme assay. The suppressive action depended on dosage.

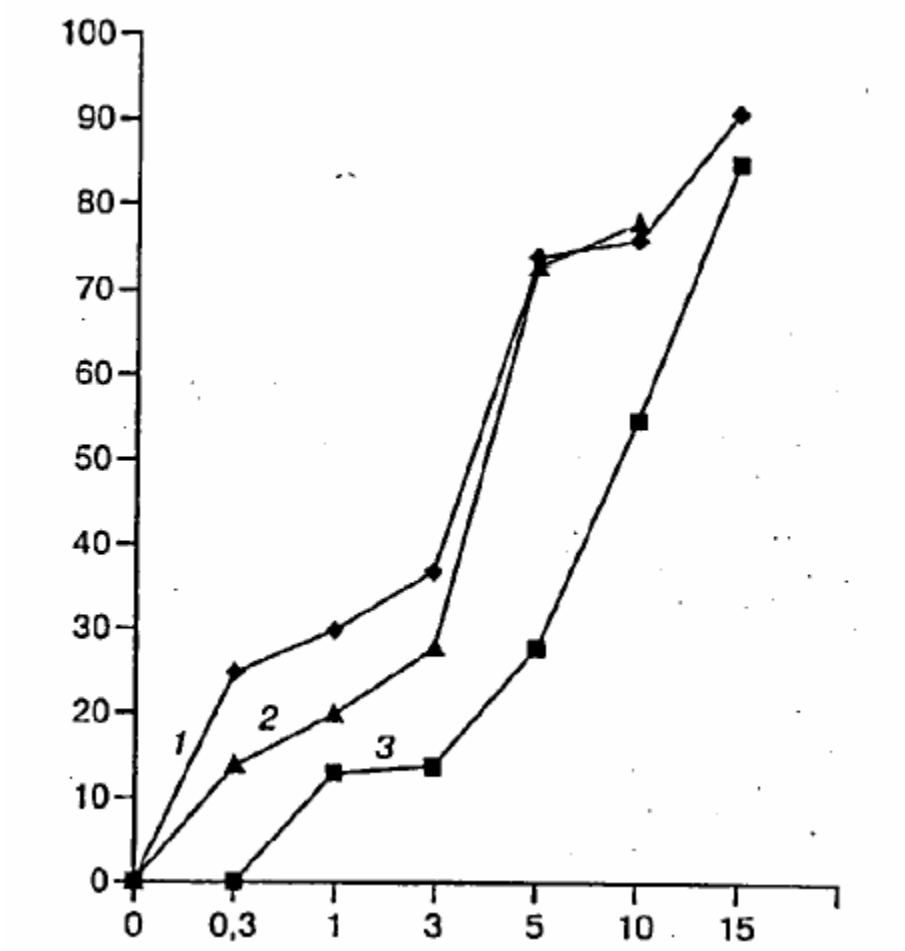

Figure 1: The influence of various concentrations of rimantadine (1), ribavirin (2), and arbidol (3) on the reproduction of influenza virus strains A/Duck/Novosibirsk/56/05 (H5N1) in SPEV cell culture. Along the ordinate – inhibition percent by OP_{450} markers. Along the abscissa – drug concentration in mcg/ml.

The variations of HPAI virus influenza A/H5N1 isolated from people in Southeast Asia showed resistance to rimantadine and amantidine. It was shown that in the resistant strains the amino acid asparagine occupied position 31 of protein M2, rather than the usual serine (announced by the WHO in 2004 on the basis of data provided by A. J. Hay). Molecular-genetic study conducted in our institute showed that the western Siberian strain A/Duck/Novosibirsk/56/05 (see GenBank data for DQ234078), as well as other viruses we isolated in 2005, contain the usual serine in position 31 of protein M2.

The above-discussed results correlate with the results of our earlier studies on rimantadine, ribavirin, and tamiflu in relation to the less pathogenic influenza strains A/H5N2-N3, isolated earlier in Siberia and the Far East [3, 5, 6, 11].

Past influenza pandemics have shown that vaccines were never available at the necessary time in sufficient quantities. Production of the vaccine requires minimum six months, and creation of a pandemic vaccine ahead of time is problematic. In light of this, with the lack of available vaccines, antiviral preparations which are widely and easily available could play a big role in a future pandemic [7, 9]. The results of our study show

the sensitivity of HPAI/H5N1 to rimantadine, amantadine, arbidol and ribavirin, and allow us to recommend these drugs for use in prophylaxis and treatment.

Bibliography

1. *Guskova T. A., Nikolaeva I. S., Peters V. V.* Metodicheskiye ukazaniya po izucheniyu protivovirusnoi aktivnosti farmakologicheskogo veshchestv (Method findings in the study of antiviral effects of pharmaceutical preparations). M., 2000: 272-274.
2. *Leneva I. A., Fadeeva N. I., Fedyakina I. T.* et al. Primenenie immunofermentnoi indikatsii virusspesificheskikh v izuchenii novogo protivogrippoznogo preparata arbidol. (Looking at virus-specific immunoenzyme indications in the study of the new antiviral drug arbidol.) Khim.-Farm. J. 1994; 9: 4-8.
3. *Lvov D. K., Yamnikova S. S., Fedyakina I. T.* et al. Ekologiya i evolutsiya virusov grippa v Rossi (Ecology and evolution of the influenza virus in Russia), 1979 – 2002. Vopr. Virus., 2004; 3: 17-24.
4. *Lvov D. K., Shchelkanov M. Yu., Deryabin P. G.*et al. Izolatsiya shtammov virusa grippa A/H5N1 ot domashnykh i dikikh ptits v period epizootii v zapadnoi sibirii (iyul 2005) i ikh deponirovaniye v gosudarstvennuyu kollektsiyu virusov RF (The isolation of strains of the influenza A/H5N1 virus from domestic and wild fowl during the period of epizootic in western Siberia in July 2005, and their categorizing in the government collection of the Russian Federation) – August 8, 2005. Vopr. Virus., 2006; 1:11-14.
5. *Fedyakina I. T, Yamnikova S. S., Galegov G. A., Lvov D. K.* Deistviye ofitsialnykh protivovirusnykh preparatov na reproduktsiyu virusov grippa ptits A/H5, izolirovannykh v Rossii (The effect of official antiviral preparations on the reproduction of avian influenza virus A/H5, isolated in Russia). Vopr. Virus., 2005; 4: 35-37.
6. *Fedyakina I. T, Leneva I. A., Yamnikova S. S.* et al. Chuvstvitelnost virusov grippa A/H5, izolirovannykh ot dikikh ptits no territorii Rossii, k arbidolu v kulture kletok MDCK (The sensitivity of influenza virus A/H5, isolated from wild birds in Russia, to arbidol in MDCK cell culture). Vopr. Virus., 2005; 6: 32-35.
7. Avian Influenza: Assessing the Pandemic Threat. WHO/CDC report, January 2005.
8. *Fock R., Bergman H., Bubmann H.* et al. Influenza pandemic: preparedness planning in Germany. Eurosurveillance, 2002, 7: 1-5.
9. *Gani R., Hughes H., Fleming D.* et al. Potential impact of antiviral drug use during influenza pandemic. Emerg. Infect. Dis., 2005; Vol. 11 No. 9: 1355-1362.
10. *Guan Y., Poon L. L., Cheung C. Y.* et al. H5N1 influenza: a protean pandemic threat. Proc. Natl. Acad. Sci. USA, 2004; Vol. 101 No. 21: 8156-8161.
11. *Lvov D.K., Yamnikova S. S., Fedyakina I. T.* et al. Evolution of H4, H5 influenza A viruses in natural ecosystems in Northern Eurasia (2000-2002). Proceedings of the International Conference on Options for the Control of Influenza (Okinawa, Japan, October 7-11, 2003). International Congress Series, 2004; 1263: 169-173.
12. *Webby R. J., Webster R. G.* Are we ready for pandemic influenza? Science, 2003; 302: 1519-1522.

Published 10/31/05