EFFECT OF INFLUCID IN VITRO AGAINST THE PANDEMIC STRAIN 2009 A(H1N1) "SWINE" ("MEXICAN") FLU

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ABSTRACT

The global development of the flu pandemic of the new variant subtype A(H1N1), the so-called "swine" or "Mexican" flu, and the absence of reliable anti-flu preparations effective against all multi-form circulating strains make the search for less narrowly focussed homeopathic and phyto-preparations against flu more urgent. This paper demonstrates the effectiveness in a model system in vitro of Influcid against a standard strain of the pandemic flu A(H1N1). The activity of the preparation is mainly expressed not in direct anti-viral action, but rather in adaptive action that leads to increasing resistance of the cells to the viral cytopathogenic effect.

Key words: *pandemic flu, A(H1N1), "swine flu", Influcid.*

INTRODUCTION

The preparation, Influcid, (German Homeopathic Union – DHU) has the following composition: 100 g of the solution contains: Aconitum D3 – 10 g, Gelsemium D3 – 10 g, Ipecacuanha D3 – 10 g, Phosphorus D5 – 10 g, Bryonia D2 – 10 g, Eupatonum perfoliatum D1 – 10 g. Other ingredients: eucalyptus globules, 96 % ethanol, purified water. Alcohol content, 45 % by volume. According to data in the literature, Influcid significantly decreases symptoms present in all acute respiratory diseases such as hyperthermia, pain in the extremities, cough, mucus membrane hyperimia and inflammatory processes in the pharynx and larynx (1).

Formerly, we investigated the anti-viral effect of Influcid *in vitro* with respect to a panel of viruses of type A bird flu (H5N1; H5N2; H7N3; H9N2) and the standard strains of human flu A(H3N2), A(H1N1) and B. A study was also done on the effect of the preparation *in vivo* on mice infected with a lethal dose of strain A/PR8/34 (H1N1) adapted to this model (2).

At present, we are witnesses to the spread of the first pandemic of the XXI century – the pandemic of so-called "swine" or "Mexican" flu caused by a qualitatively new strain of a subtype A flu (H1N1) – a triple reassortment that contains segments of RNA derived from the North American line of swine flu (segments HA, NP and NS), the Eurasian line of swine flu (segments NA and M), the North American line of bird origin (segments PA and PB2) and segment PB1 from seasonal flu subtype H3N2 (5). At the time this paper was written (07/07/2009), according to WHO data, there were 94,512 laboratory registered cases of "swine flu" worldwide, of which 429 were fatal. The pandemic continued mainly in the southern hemisphere which was at the time at the height of the winter season (Argentina, Australia, Chile, New Zealand). However, regardless of the summer season, in the northern hemisphere, incidence of this new flu variant continued to increase – most of all in the US where the number of cases reached almost 34,000, as well as in Mexico, Canada, Korea, Japan and Thailand (6).

According to laboratory studies, the pandemic strain A(H1N1) is resistant to adamantans (remantadine and amantadine), but is sensitive to neuraminidases (oseltamivir and zanamivir). However, information has already appeared on the isolation of the first virus of this variant resistant to oseltamivir (Tamiflu) (6). Experience of recent years indicates that it is practically impossible to select an ethiotropic antiviral preparation effective against all the multiform seasonal viruses that circulate. Thus, remantadine, in addition to the fact that it is ineffective against B flu, in recent epid-seasons did not have any effect on a significant portion of viruses of the A(H3N2) subtype. On

the other hand, viruses of the A(H1N1) subtype were in most cases sensitive to remantadine, but rapidly acquired resistance to oseltamivir. All this constitutes a basis for searching for preparations directed not toward the virus as such or its interaction with the cell but toward stimulating cell resistance, developing interferons, and immune protection. With the present unclear prospects of timely development of a pandemic vaccination against "swine" flu, the role of such preparations in the initial phase of a pandemic is especially important. This prompted us to study the possible effect in vitro of Influcid with respect to the standard strain of "swine" flu A/California/07/09 (H1N1) sw1.

MATERIALS AND METHODS

A study of the effect of Influcid done with an MDSK cell culture recommended by the WHO reference centers and by the Ministry of Public Health for Flu of the Russian Federation for isolating and studying flu viruses (3). The strain of the pandemic "swine flu" A/California/07/09 (H1N1) sw1 was graciously provided to us for the scientific studies by Prof. A. I. Klimov (Centers for Control and Prevention of Diseases (CDC), Atlanta, Georgia, US).

It turned out that for our purposes, the liquid (drop) form of Influcid was most suitable. As demonstrated previously, this preparation is characterized by complete absence of toxicity when it is dissolved at 1/50-1/400 and is in contact with cells for 3 days.

Antiviral activity of Influcid was evaluated on cells placed in standard doses on a 96-well cell culture plate. From the original virus-containing allantoic liquid, a series of ten-fold dilutions from 10^{-1} to 10^{-7} was prepared and placed in corresponding wells with the cell monolayer. After 48 hours, the results were checked for hemagglutination reaction (RHA). In the control and in the experiment, the inverse logarithm of the greatest dilution of the original virus that could cause a positive reaction of hemagglutination in a well was used as the virus titer and was expressed as 50 % of tissue infectious doses (TID_{50}). The virus inhibiting effect of the preparation was evaluated according to the decrease in virus titer in the experiment as against the control ($\Delta lgTID_{50}$). A second important indicator was the reaction of cell regeneration in cultures with MTT tetrazol stain (Thyazolyl blue), the intensity of which reflects the degree of viability of cells as a result of reduction of the stain by mitochondrial and partially by cytoplasmic dehydrogenases. This test is used frequently in virology to evaluate the cytopathogenic action of viruses on the cell (8). Its results can be interpreted as the degree of cell resistance to the effect of viruses. The microtetrazol test is also widely used for evaluating the effect on cells of toxicants, pharmacological preparations and unfavorable environmental factors (4) and for this reason, toxicity of the preparations being tested *in vitro* can be evaluated simultaneously with the antiviral effect. The preparation was added to the cell culture medium 1 hour before it was infected with the virus ("prophylactic" plan of administration) and 1 hour after infection with the virus ("treatment" plan of administration) in a dilution with a PBS buffer of 0.5 % and 1 % (concentration in the medium of the original liquid preparation).

RESULTS

When Influcid was administered according to the treatment plan (1 hour after infecting the cells), the preparation moderately suppressed the production of virus particles which was evident in the hemagglutination reaction – at a concentration of 1 %, the decrease of hemagglutinating activity ($\Delta lgTID_{50}$) was 1.0; with a dose of 0.5 %, it was 0.5, that is, the infectious activity of the virus dropped by a factor of 10 and 6.7, respectively. The results of the comparative study of the effect of the preparation according to the given criterion with respect to a panel of human and bird flu viruses are presented in Table 1. The greatest activity of Influcid in this study was apparent with respect to standard A and B strains of human flu virus isolated in different years and was comparatively low or absent with respect to a number of mutants of bird flu.

Evaluation of MTT regeneration disclosed a significant decrease in cytopathic activity of the virus in both the "prophylactic" plan (Fig. 1 and 2) and the "treatment" plan (Fig. 3 and 4) of administering the preparation. In the "prophylactic" plan, the effect of the preparation was dose-dependent (Fig. 1) – Influcid in a concentration of 1 % had a reliably greater effect than at 0.5 %.

The effect was best expressed in the zone of high virus titers (-1 and -2 log or 10^4 and 10^3 TID_{50}), while in concentrations of 1 %, the preparation almost completely blocked the cytopathic reaction of the cells in the culture even at the highest infectious dose of the virus: 10^4TID_{50} (Fig. 2). Influcid also reliably decreases the cytopathic effect of the virus in the "treatment" plan of administration (Fig. 3 and 4), and, in this case, the effect of the preparation in doses of 0.5 % and of 10 % was approximately the same, but reliably different from the control (Fig. 4).

DISCUSSION

When comparing the data obtained with the MTT method with the results recorded with the RHA method, it is evident that with a highly pathogenic infection with the strain of "swine" flu A/California/07/09sw1, Influcid has a positive effect mainly by decreasing cytopathic reactions of the cells to the virus, that is, it increases the resistance of the cells themselves. In this sense, its effect may be considered not so much as being directly antiviral but as being adaptive, and the result of this is a reinforcement of the resistance of the organism to the viral infection.

Comparison of the data on activity of the preparation with respect to various standard strains of the flu virus shows that its effect on RHA against the pandemic strain A(H1N1) is relatively slight – most of the activity seemed to pertain to strains A(H3N2) and B. The effect against the standard vaccine flu strain A(H5N1)-NIBRG-14 was also slight (Table 1). It should be noted, however, that in this comparative study, the method for evaluating the cytopathic effect of viruses on MTT regeneration by the infected cells was not used so all possible aspects of the effect of the preparation may not have been disclosed. Nevertheless, the importance even of the relatively slight positive effect of Influcid against strains A(H5N1) must be stressed since the threat of an epidemic of highly pathogenic "bird" flu should not be removed from the agenda regardless of the onset of the "swine" flu pandemic since reports of cases of A(H5N1) flu with a fatality rate exceeding 50 % appear constantly in developing countries (7).

Finally, regardless of the simplicity and nature of the models in vitro, what should be noted in the study of homeopathic preparations, which, of course, act mainly at the systemic level, is their limitations. For this reason, earlier we also confirmed the antiviral effect of Influcid on an animal model – white mice infected with lethal and sublethal doses of a widely known strain A/PR/8/34 (H1N1) (2). The preparation diluted with a physiological solution was administered to the mice intraventricularly once a day for 4 days immediately after infection with a pharmacologically appropriate dose. According to the index of protection (IP) criterion and considering the demise of experimental animals in all periods and with both infectious doses of the virus of 1LD50 and 0.1LD50, we obtained IP = 41.2 %, and with the infectious dose of the virus of 0.1LD50, IP = 75 % (the preparation is considered active if the IP ≥ 40 %). Thus, the

Table 1: Comparative antiviral activity of Influcid in vitro on strains of various subtypes of the flu virus as evaluated by the RHA method

No.	Virus strain	Antiviral effect in $\Delta lgTID_{50}$
1	A/N.Caledonia/20/99 (H1N1)*	1.5
2	A/Victoria/35/72 (H3N2)*	2.0
3	A/Wisconsin/67/05 (H3N2)	1.0
4	B/Malaysia/2506/04*	2.0
5	A/NIBRG-14 (H5N1)*#	1.0
6	A/duck/Potsdam/1402/6/86 (H5N2)+	0.5
7	A/mallard/NT/12/02 (H7N3)+	0
8	A/HongKong/1073/99 (H9N2)+	0
9	**A/California/07/09 (H1N1)***##	0.75

Note: * – human flu viruses; *# – vaccine strain A(H5N1); *## – pandemic virus A(H1N1) (virus of "swine" or "Mexican" flu) causing a flu pandemic developing since April 2009; + – bird flu virus.

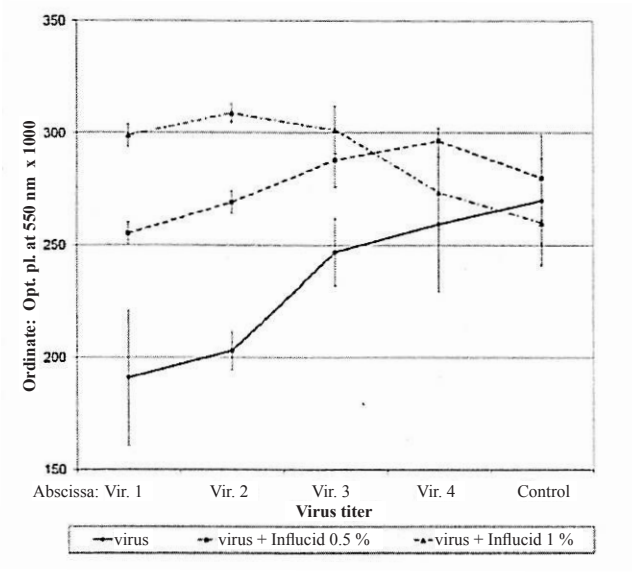

Fig. 1. Antiviral effect of Influcid with respect to the virus of "swine flu" A/California/0709 (H1N1) sw1 in MDCK cell culture. Preincubation with the preparation for 1 hour, infection with the virus, assessment of results after 24 hours. Test method: MTT.

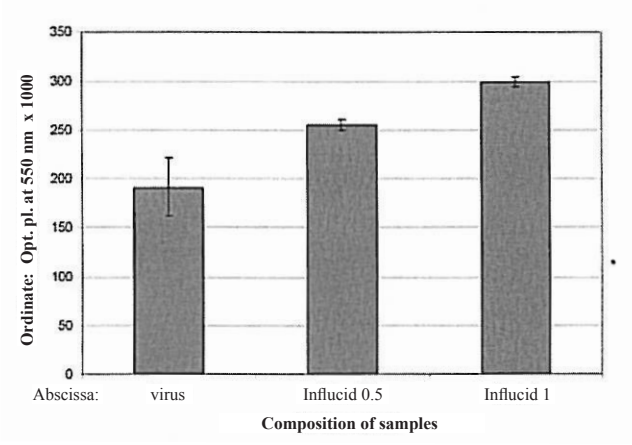

Fig. 2. Antiviral effect of Influcid with respect to "swine flu" virus A/California/07/09 (H1N1)sw1. Preincubation with the preparation for 1 hour. Test method: MTT, Data with a virus dose of 10^4TID_{50}.

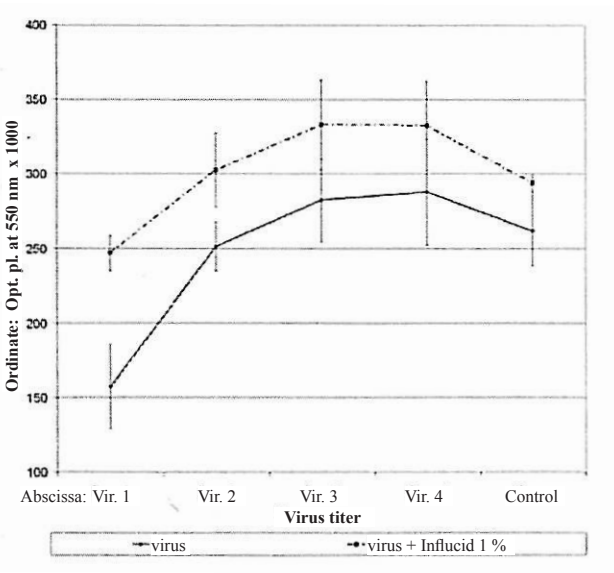

Fig. 3. Antiviral effect of Influcid with respect to "swine flu" virus A/California/07/09 (H1N1)sw1 with preliminary infection of MDCK cells with virus (1 hour before administering the preparation). Test method: MTT.

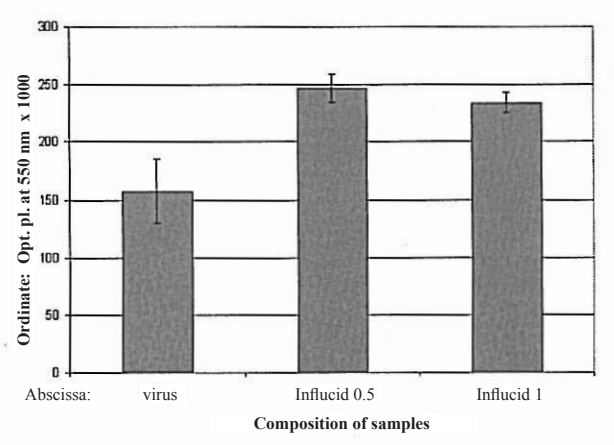

Fig. 4. Antiviral effect of Influcid on "swine flu" virus in an MDCK cell culture with preliminary infection with virus 1 hour before administering the preparation. Virus dose: 10^4TID_{50}.

effectiveness of the preparation was demonstrated both on the whole and specifically for an 0.1LD50 infectious dose of the virus (titer of the virus 10-2) when IP >> 40 %. Statistical processing with non-parametric methods (Wald-Wolfowitz Runs Test, program Statistica for Windows 6.0) also indicated the reliable effect of Influcid *in vivo* (for a virus dose of 0.1LD50, P = -3.676; p = 0.000237). A convincing result was also obtained in processing with the method of regressive analysis (plotting of linearized dependence of animals surviving on the time after infection).

CONCLUSIONS

1. Influcid is effective as an adaptogenic anti-flu preparation *in vitro* in a culture of MDCK cells infected with "swine" flu virus (new pandemic strain A(H1N1).
2. The activity of the preparation is manifested both in "prophylactic" and in "treatment" plans of administration.
3. The positive effect of Influcid was demonstrated mainly in a test of MTT cell regeneration, which points to the cellular mechanism of the antiviral effect expressed in increased resistance to the cytopathic effect of the virus.

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