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ACTION OF REMANTADINE ON FUSION OF THE LIPID ENVELOPE OF INFLUENZA A VIRUS WITH PLASMA AND INTERNAL MEMBRANES IN LYMPHOBLASTOID CELLS

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For transcription of the virus genome to begin in cells infected by enveloped viruses, the nucleocapsid of the virus must be released from external proteins and the lipid envelope. For this purpose, the influenza virus utilizes the path of receptor-mediated endocytosis: it is bound by receptors on the plasma membrane, and penetrates through covered pits and vesicles into the endosomes, where at low pH fusion of the lipid envelopes of the virus and endosomes takes place, leading to release of the nucleocapsid [4, 11]. Fusion of the lipid envelope of the virus with cell membranes was studied until very recently by inducing this process artificially by lowering the pH, and thus observing only fusion of the lipid envelope of the virus and the plasma membranes of the cells [8, 14]. By the fluorescence quenching method [12] the fusion of the lipid envelope of the virus both with plasma membranes of cells and with membranes of endosomes can be evaluated quantitatively. In this method a fluorescent probe with hydrocarbon chain, determining incorporation of the probe molecule into the lipid envelope of the virus, is used. If the virus membrane fuses with the cell membranes, redistribution of the probe on the membrane takes place, its concentration is reduced, quenching is reduced, and accordingly fluorescence increases, and an effect of dequenching of fluorescence (DQF) is observed.

Remantadine is widely used nowadays in the chemotherapy of influenza, but its mechanism of action has not yet been explained [1]. According to one group of investigators remantadine is a substance which, like NH_4Cl and chloroquine, inhibits fusion of the lipid envelope of the virus and endosomes. On the other hand, there is evidence [6, 7] that remantadine blocks the stage of RNP release from the nucleocapsid, interfering with the beginning of transcription. The aim of the present investigation

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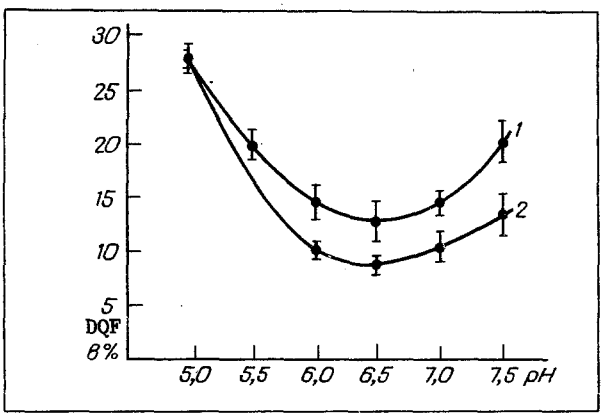

Fig. 1. Dependence of fusion of lipid envelope of influenza virus with cell membranes on pH: 1) Namalwa cells; 2) Raji cells.

was to study the effect of remantadine on fusion of the lipid envelope of the virus, both with plasma membranes of cells and with endosomal membranes.

EXPERIMENTAL METHOD

A remantadine-sensitive strain of influenza virus A/Krasnodaar/101/59(H2N2), grown in the allantoic cavity of 11-day chick embryos, concentrated and purified by the method in [9], was used. Cell-suspension lymphoblastoid lines Namalwa and Raji were grown on medium RPMI-1641 with 10% fetal serum and 20 mM glutamine. Before the work the cells were washed to remove serum 3 times with Hanks' solution, without phenol red.

The fluorescent probe was synthesized by mixing 100 mg of stearylamine, dissolved in a chloroform—methanol (4:1) mixture, with 100 mg of rhodamine isothiocyanate B in the same solution. After incubation at 37°C for 18 h the reaction was stopped by the addition of 100 mg stearylamine. Conjugate of rhodamine isothiocyanate B with stearylamine (RITCB—SA) was isolated and purified by the method in [2]. The product obtained was estimated by mass-spectrometry. Virus labeled with RITCB—SA was obtained as in [12]. In experiments with endocytosis of influenza virus, 10 μg of virus labeled with RITCB—SA was incubated with 10^7 Raji or Namalwa cells in 200 μl of 10 mM phosphate buffer, pH 7.4. After incubation at 4°C for 10 min, necessary for adsorption of the virus, the mixture was heated to 37°C. Preliminary kinetic experiments showed that fusion of the membranes of virus and cells is largely complete after 1 h, and for that reason in the subsequent experiments the reaction mixture was incubated for that time. The reaction was stopped with 1 ml of cold phosphate buffer (100 mM), pH 7.4, thanks to which the same pH value was established in all the samples, the samples themselves were placed in ice, and the values of fluorescence (F) were measured at λ_{ex} = 550 nm and λ_{em} = 575 nm. In the control, fluorescence of a preparation of virus labeled with RITCB—SA in the absence of cells was used as the control (F_0). The intensity of fluorescence was then determined after addition of Triton X-100 to the samples up to 0.5% (F_{100}). This value was regarded as the 100% value of dequenching of fluorescence (DQF or "dequenched" virus). The percentage of DQF was calculated by the equation:

$$P\Phi = \frac{\Phi - \Phi_0}{\Phi_{100} - \Phi_0} \cdot 100\ \%.$$

EXPERIMENTAL RESULTS

In the experiments of series I the effect of pH on DQF was studied during interaction of influenza A/Krasnodar/101/59 virus with Namalwa and Raji lymphoblastoid cells. After adsorption of the virus at 4°C for 10 min, the required pH was established with 0.1 M phosphate buffer, and the sample incubated at 39°C for 1 h. The data in Fig. 1 show that with an increase in pH to 7.4 or a decrease in pH to 5.0 the value of DQF rises. Incubation of the virus for 1 h at 4°C, when the process of endocytosis was blocked, had virtually no effect on DQF. Addition of NH_4Cl (50 mM) and monensin (10^{-5} M), inhibitors of endocytosis, to the reaction mixture at pH 7.4 led to a fall of the value of DQF by 12 and 18%, respectively. Addition of NH_4Cl at pH 5.0 did not affect DQF.

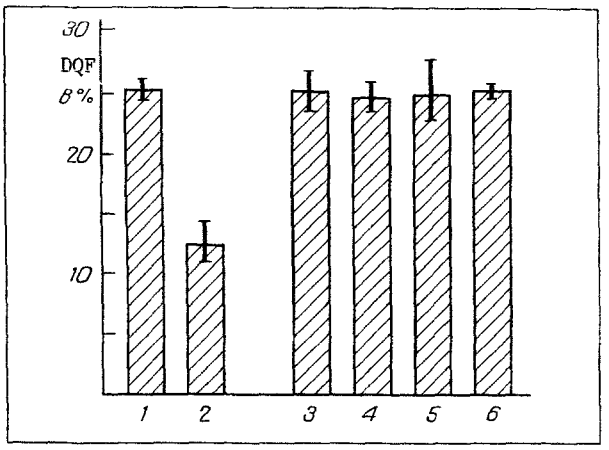

Fig. 2. Effect of remantadine on fusion of lipid envelope of influenza A/Krasnodar/101/59 virus with Namalwa cell membranes. 1, 2) pH 5.0. 1) Virus, 2) virus + remantadine (25 μg/ml), 3-6) pH 7.4. 3) Virus, 4) virus + remantadine (50 μg/ml), 5) virus + remantadine (500 μg/ml), 6) virus + remantadine (1 mg/ml).

TABLE 1. Localization of Virus Lipid Envelope during Interaction of Influenza A/Krasnodar/101/59 Virus with Raji Cells at pH 5.0 and pH 7.4

pH	KI		$NiCl_2$	
	EV, %	PV, %	EV, %	PV, %
5,0	11±2	62±2	4±4	50±6
7,4	20±4	0±1	22±2	18±1

Legend. EV) Fraction of virus whose envelope was fused with intracellular (endosomal) membranes. PV) Fraction of virus whose envelope was fused with the cell plasma membrane. For calculation of EV and PV, see text. Results with $NiCl_2$ must be regarded as qualitative, for $NiCl_2$ solution gives rise to appreciable absorption of exciting and fluorescent radiation, as well as to cell aggregation.

In the experiments of series II the localization of the virus membranes was studied after fusion with cell membranes, using fluorescence quenchers which do not penetrate into cells ($NiCl_2$ and KI). Preliminary experiments with "dequenched" and original labeled virus showed that in a concentration of 0.5 M the above-mentioned quenchers inhibited fluorescence by 80%, i.e., the fraction of suppressed fluorescence was 0.8 and the residual fraction 0.2. On the assumption that the intracellular virus was completely "dequenched," the relative percentage of virus envelope in the intracellular, presumably endosomal (EV %) and plasma PV %) membranes, was estimated. $EV = 100\% \times [(F_q - 0.2\ F)/0.8]/F_{100}$, where F_q denotes fluorescence in the presence of the quencher, and PV % = DQF % − EV %. The results in Table 1 show that at pH 5.0 virtually all the virus fused with membranes was located in the plasma membrane, but at pH 7.4, most of the virus was located in the endosomal membranes.

The results of the experiments of series I and II show that at pH 5.0 DQF is due to fusion of the lipid envelopes of the virus and the plasma membranes of the cells, whereas at pH 7.4 it is due to fusion of the lipid envelopes of the virus and intracellular endosomes. Thus the DQF method which we used can clearly distinguish these processes and can be used to study the effect of remantadine on each of them. Different concentrations of remantadine (50-1000 μg/ml) at pH 4 in the reaction medium had no effect on the value of DQF, but conversely, incubation of the virus with the cells in the presence of 25 μg/ml of remantadine at pH 5.0 depressed the value of DQF almost by half (Fig. 2).

It can be concluded from these results that remantadine inhibits fusion of the lipid envelope of the virus with plasma membranes, whereas it does not act on fusion of the lipid envelope of the virus with endosomes. This conclusion is in agreement with electron-microscopic data [5] on interaction of influenza virus with HeLa and Vero cells, showing that, when penetrating into zero cells, the influenza virus utilizes the path of receptor-mediated endocytosis. In HeLa cells the lipid envelope of the virus fuses with plasma membranes, but remantadine inhibited reproduction of the virus in them, whereas it had no effect on reproduction in zero cells. Our results contradict those obtained by other workers [10, 1], who concluded, on the basis of the fact that remantadine is an amine, that it renders the contents of the endosomes alkaline, and they explain its inhibitory action on reproduction of influenza virus by this process. There is evidence [13] that for remantadine to be active, it must be present in the extracellular medium, but on the other hand, it has been shown [3] that remantadine can induce modification of the cell plasma membrane. This suggests that remantadine is incorporated into the plasma membranes of cells, and that its effect on the stage of fusion of the lipid envelope of the virus and plasma membranes can be explained in this way.

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