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A new class of antivirals: antisense oligonucleotides combined with a hydrophobic substituent effectively inhibit influenza virus reproduction and synthesis of virus-specific proteins in MDCK cells

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To enhance the penetration of oligonucleotide ('oligo') into cells, the oligo was combined with the hydrophobic undecyl residue. Using the 'DNA-synthesator', we synthesized oligo, complementary to the loop-forming site of the RNA, encoding polymerase 3 of the influenza virus (type A), and combined it with the undecyl residue added to the 5' terminal phosphate group. It was found that the modified oligo effectively suppresses the influenza A/PR8/34 (H1N1) virus reproduction and inhibits the synthesis of virus-specific proteins in MDCK cells. Under the same conditions, the non-modified antisense oligo and modified nonsense oligo did not affect the virus development.

Antisense; Oligonucleotide; Influenza; Virus; Inhibition; Translation

1. INTRODUCTION

Modulation of the ability of biopolymers to penetrate biological membranes seems to have become one of the key problems of modern biochemistry and medicine. The difficulties connected with the passing across the membrane barrier limit considerably the possibilities of drug targeting [1], genetic transformation of cells [2], regulation of intracellular processes by exogenous protein factors [3] and nucleic acids [4].

The method for imparting transmembrane properties to water-soluble proteins, based on their covalent modification with hydrophobic 'anchor' groups (e.g. fatty acid residues) has been developed recently [5-7]. The modified protein molecules acquire the ability to translocate across lipid membranes [5] and to penetrate into intact cells [6,7].

The principle of artificial hydrophobization [5] permitted us to suggest a method for the suppression of virus reproduction in the cells: fatty acylated antiviral antibodies considerably inhibit the virus development in cells, presumably, disturbing the proper assemblage and budding of infective virus particles [8,9]. However, it is likely that the hydrophobized antibodies do not affect the translation of the virus-specific proteins in the infected cells (in preparation).

In order to block the synthesis of virus components in a cell it is reasonable to use the antisense inhibition of this process by short complementary oligonucleotides ('oligos'), which are regarded now as one of the most promising tools for the regulation of transcription and translation [4,10]. However, the application of oligos is considerably limited by the low efficiency of their penetration into intact cells.

For enhancing the oligos action on cells we suggested [11] that they should be terminally modified with hydrophobic groups. In the present work we have studied the effect of antisense oligo, modified at the 5'-end with fatty radicals, on the reproduction of the influenza A/PR8/34 (H1N1) virus and synthesis of the virus-specific proteins in MDCK cells.

2. MATERIALS AND METHODS

2.1. *Cells and viruses*

MDCK cells were kindly presented by the Laboratory of Cell Cultures (Institute of Virology, USSR Academy of Medical Sciences). Cells were grown as a monolayer stationary culture in 199 medium, containing 10 mM Hepes, 0.075% sodium bicarbonate, 10% fetal calf serum and 200 μg/ml gentamycin.

Influenza virus (strain A/PR8/34 (H1N1)) was passed in the allantoic cavity of 9-day-old fertilized eggs at low multiplicity. The allantoic fluid was clarified at 6000 × g × 15 min, stored at −70°C, and then used as an inoculum in virus reproduction experiments.

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Abbreviations: Aerosol OT, sodium bis(2-ethylhexyl)sulfosuccinate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid sodium salt; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PFU, plaque forming units

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2.2. *Oligo synthesis*

The oligos were synthesized with the DNA-synthesator (Applied Biosystems, Model 380 B-02) using 2-cyanoethylphosphoramidite building blocks (American Bionetics) with a cycle modified for a 3 mmol scale [12].

2.3. *Introduction of undecyl radicals during oligo synthesis*

n-Undecyl-2-cyanoethyldiisopropylamidophosphine was synthesized from undecanol by a modified 3-stage procedure with the application of 2-cyanoethyl-*N,N,N',N'*-tetraisopropylphosphorodiamidite [13].

This reagent was introduced at the 5'-end of the growing chain during the oligo synthesis with the DNA-synthesator. This last stage was repeated twice without deblocking and capping.

The modified oligos were precipitated in $LiClO_4$-acetone and were analyzed under denaturing conditions (7 M urea) by PAGE.

2.4. *Oligo modification in reversed micelles*

The oligos, containing a 5'-end phosphate group, were obtained by the addition of bis(2-cyanoethyl)diisopropylamidophosphine [14] at the last stage of the oligo synthesis with the DNA-synthesator.

The procedure of modification of these oligos with undecyl radicals in the system of Aerosol OT reversed micelles was analogous to the method of protein modification described earlier [15,16]. Mes buffer (550 μl, pH 5.5), containing 10 nmol of oligo, were solubilized in 8.5 ml of 0.3 M Aerosol OT (Merck) solution in octane. 220 mg of dry 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide (Sigma) were added to the obtained optically transparent system of reversed micelles. After 30 min incubation, 50 μl of undecanol (Sigma) were solubilized in the reaction system.

The system obtained was incubated for 10 h at room temperature and then the modified oligo was precipitated by 30 ml of cold (0°C) acetone. The precipitate formed was then removed by centrifugation and thoroughly washed 2 times with 30 ml cold acetone. The residual acetone was removed using a rotor evaporator.

The modified oligos were analyzed by PAGE as described above.

2.5. *Virus reproduction*

The monolayers of permissive MDCK cells were infected with influenza virus (strain A/PR8/34) at 20–100 plaques per dish multiplicities. After adsorption (60 min, 37°C) the virus inoculums were removed, the cells were washed and covered by the standard agar overlay with trypsin [17], containing different concentrations of oligos. Three days after infection the cells were stained, the number of plaques was counted and their size was estimated.

2.6. *Synthesis of virus-specific proteins*

The MDCK cells were infected with influenza A/PR8/34 virus at high multiplicity (10 PFU/cell). After removing the virus inoculum and washing the cells, the equimolar mixture of the ^{14}C-labelled (5 μCi/ml) amino acids (UVVVR, Czechoslovakia) in 199 medium, containing 100 μM of oligos, was added to the infected cells. Fifteen hours after the infection, cells were collected, washed (2 times with cold 7% trichloroacetic acid and 2 times with 96% ethanol) and lysed with the buffer for electrophoresis. The cellular lysates were analyzed by PAGE. The gels were fixed, stained and dried and the autoradiographs were made using R-Film.

3. RESULTS

3.1. *Oligo synthesis and modification*

Two oligos were synthesized using the standard technique, namely,

TTGACGAAATT (I)

which is complementary to the loop-forming site of the RNA, encoding polymerase 3 of the influenza virus [18]; and a 'nonsense' oligo

GGGTGTTAGAG (II)

which does not interact with influenza virus RNAs.

For the introduction of hydrophobic radicals at the 5'-ends of these oligos, two different methods were developed.

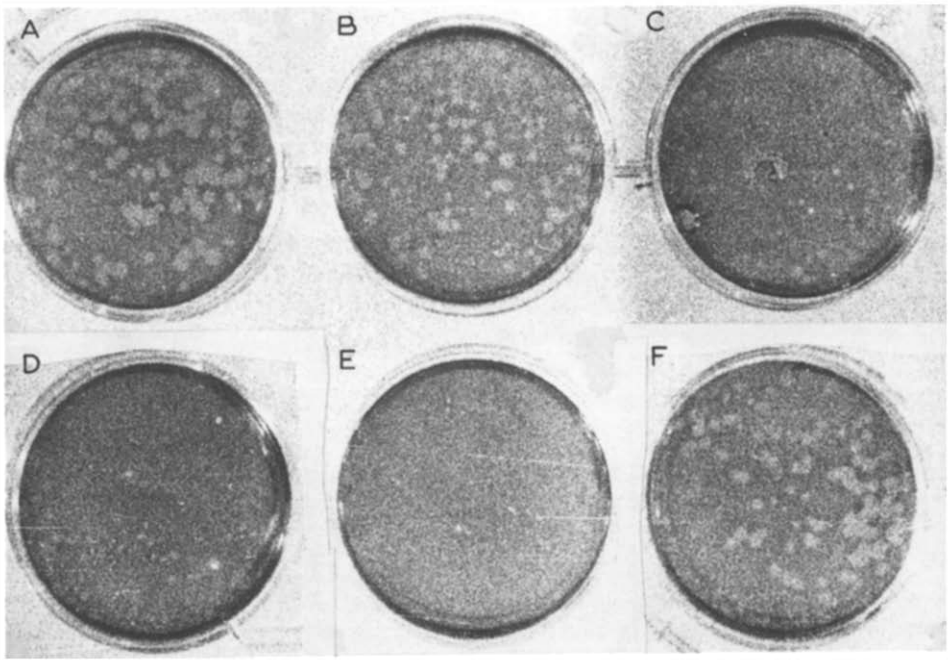

Fig.1. Plaque formation in the monolayer of MDCK cells infected by influenza A/PR8/34 virus [17] in the media containing: (A) no oligos; (B) 10 μM, (C) 50 μM, (D) 100 μM, (E) 200 μM of modified oligo (I); (F) 200 μM of non-modified oligo (I).

According to one of them the *n*-undecanol derivative *n*-undecyl-2-cyanoethyldiisopropylamidophosphine

$$n\text{-}C_{11}H_{23}O\text{-}P\begin{matrix}\diagup N(iPr)_2\\ \diagdown O(CH_2)_2CN\end{matrix} \quad \text{(III)}$$

was introduced at the 5′-ends of the growing chains at the last stage of the oligo synthesis with the DNA-synthesator. By the PAGE data the yield of the undecyl-modified oligos at this last stage was 90–95%.

The second method, which is most valuable for the hydrophobization of already formed (e.g. natural) oligos, is based on the use of the system of the surfactant reversed micelles in the organic solvent (Aerosol OT in octane) [19] as a medium for the oligo modification. Being solubilized in such a system the polar oligo molecules are entrapped in the inner water cavity of reversed micelles. The modifying water-insoluble reagent is localized not only in the bulk phase of the organic solvent, but can be incorporated in the reversed micelle, thus coming into contact with the oligo groups to be modified [15,16].

We applied this method for the attachment of undecanol residues to the oligos, containing 5′-end phosphate groups, activated by a standard carbodiimide procedure. In this case, the yield of the obtained undecyl-modified oligos was 60–70%.

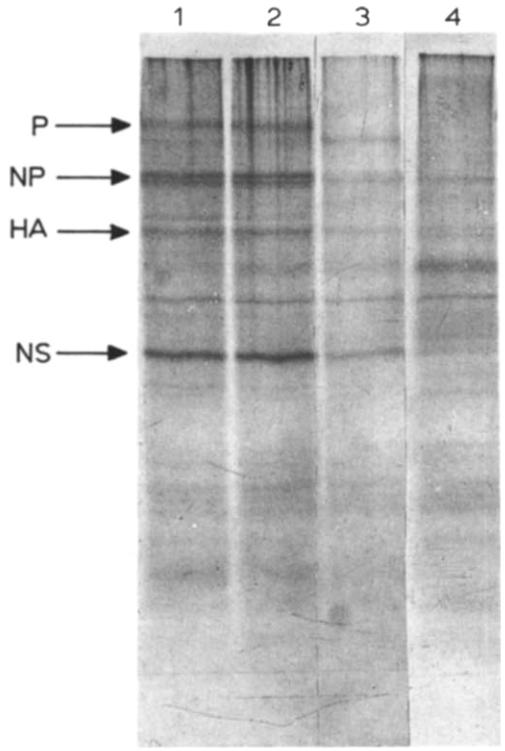

Fig.2. Protein synthesis in the (1–3) infected and (4) non-infected MDCK cells. The cells were incubated in the media, containing: (1,4) no oligos; 100 μM of (2) non-modified and (3) modified oligo (I). Arrows indicate the influenza virus polymerases (P), nucleoprotein (NP), hemagglutinin (HA) and non-structured (NS) proteins [20].

3.2. *Oligos affect virus reproduction*

The effect of the undecyl-modified oligos on the reproduction of influenza A/PR8/34 virus in permissive MDCK cells was investigated using the plaque-assay method.

As can be seen in fig.1, the modified oligo (I) considerably suppressed the development of the virus infection (the number and the size of the plaques), whereas the non-modified oligo (I) did not produce such an effect at the same concentrations.

The modified nonsense oligo (II) did not affect the virus reproduction, i.e. the observed effect resulted from the specific interaction of antisense oligo (I) with the corresponding site of viral RNA (not shown in the figure).

Fig.2 illustrates the results of the experiment studying the protein synthesis in high-multiplicity infected cells. Four major bands, corresponding to the virus-specific proteins [20], are observed at the autoradiograph of the infected MDCK cells lysate (lane 1). The treatment of these cells with non-modified oligo (I) practically did not affect the synthesis of viral proteins (lane 2).

On the contrary, the addition of the modified oligo (I) to the infected cells led to a considerable inhibition of the synthesis of these proteins. In this case (lane 3) the viral polymerases are undetectable and the bands of other viral proteins are at least strongly diminished. In other words, the observed protein pattern of the cell lysate is almost similar to that, observed for non-infected MDCK cells (lane 4).

The nonsense oligo (II) (both the non-modified and the modified species) did not affect the synthesis of virus-specific proteins.

4. CONCLUDING REMARKS

The data obtained indicate that the observed inhibition of the influenza virus reproduction and synthesis of the virus-specific proteins in MDCK cells treated with undecyl-modified oligo (I) results from the intracellular binding of this oligo to the complementary loop-forming site of the viral RNA, which presumably is responsible for the RNA-protein interaction.

Convincing evidence was obtained that the covalent attachment of fatty radicals to the oligo molecule considerably enhances its action on the cells. If compared with other methods of enhancing the oligos' penetration into the cell, and increasing their activity [10,21] the method suggested in this work seems to become very promising in drug design, since the fatty modified oligos are produced with a high yield with application of simple and standard procedures, e.g. directly in the DNA-synthesator.

The key problem of oligos targeting to the infected cells must be solved for their successful medical application.

It has been recently demonstrated [22] that the effi-

ciency of the DNA penetration into the cells can be greatly increased through its incorporation into the soluble interpolyelectrolyte complexes (IPC) with carbochain polycations. Such complexes form spontaneously on mixing the nucleic acid and polycation solutions. Being entrapped in IPS, the oligo molecule acquires resistance against cleavage with nucleases, particularly in blood serum (in preparation). A target-recognizing molecule (e.g. the antibody against virus antigen) can bind covalently to the IPC-forming polycation. This might be a way to address the oligo molecules, packed in IPC particles, into a certain target cell.

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REFERENCES

[1] Pastan, I., Willingham, M.C.W. and Fitzgerald, O.J.P. (1986) Cell 47, 641–648.
[2] Smith, H.O. and Danner, D.B. (1981) Annu. Rev. Biochem. 50, 41–68.
[3] Brady, R.O. (1975) Ann. Int. Med. 82, 257–261.
[4] Inoye, M. (1988) Gene 72, 25–34.
[5] Kabanov, A.V., Nametkin, S.N., Levashov, A.V. and Martinek, K. (1985) Biol. Membrany 2, 985–995.
[6] Kabanov, A.V., Levashov, A.V., Alakhov, V.Yu., Kravtsova, T.N. and Martinek, K. (1989) Collect. Czech. Chem. Commun. 54, 835–837.
[7] Alakhov, V.Yu., Kabanov, A.V., Batrakova, E.V., Koromyslova, I.A., Levashov, A.V. and Severin, E.S. (1989) Biotechnol. Appl. Biochem. 11, in press.
[8] Kabanov, A.V., Ovcharenko, A.V., Melik-Nubarov, N.S., Bannikov, A.I., Alakhov, V.Yu., Kiselev, V.I., Sveshnikov, P.G., Kiselev, O.I., Levashov, A.V. and Severin, E.S. (1989) FEBS Lett. 250, 238–240.
[9] Kabanov, A.V., Ovcharenko, A.V., Melik-Nubarov, N.S., Bannikov, A.I., Lisok, T.P., Klushnenkova, E.V., Cherchenko, N.G., Alakhov, V.Yu., Kiselev, V.I., Sveshnikov, P.G., Kiselev, O.I., Levashov, A.V. and Severin, E.S. (1990) Biomed. Sci. 1, in press.
[10] Toulmé, J.-J. and Hélène, C. (1988) Gene 72, 51–58.
[11] Kabanov, A.V., Vinogradov, S.V., Ovcharenko, A.V., Krivonos, A.S., Melik-Nubarov, N.S., Kiselev, V.I. and Severin, E.S. (1989) Coll. Czech. Chem. Commun. 54, N 12.
[12] Oligonucleotide Synthesis: A Practical Approach (Gait, M.G. ed.) IRL, London, 1984.
[13] Nielsen, J., Taagaard, M., Marugg, J.E., Von Boon, J.H. and Dahl, O. (1986) Nucleic Acids Res. 14, 7391–7403.
[14] Uhlmann, E. and Engels, J. (1986) Tetrahedron Lett. 27, 1023–1026.
[15] Levashov, A.V., Kabanov, A.V., Khmelnitsky, Yu.L., Berezin, I.V. and Martinek, K. (1984) Dokl. Akad. Nauk SSSR 278, 246–248 (Russ.); DAN (Engl. edn) 295–297 (1985).
[16] Kabanov, A.V., Levashov, A.V. and Martinek, K. (1987) Ann. NY Acad. Sci. 501, 63–66.
[17] Zhirnov, O.P., Ovcharenko, A.V. and Bukrinskaya, A.G. (1982) Arch. Virol. 71, 177–183.
[18] Kaptain, J.S. and Nayak, D.P. (1982) J. Virol. 42, 55–63.
[19] Martinek, K., Klyachko, N.L., Kabanov, A.V., Khmelnitsky, Yu.L. and Levashov, A.V. (1989) Biochim. Biophys. Acta 981, 161–172.
[20] Zhirnov, O.P. and Bukrinskaya, A.G. (1981) Virology 109, 174–179.
[21] Leonetti, J.P., Rayner, B., Lemaitre, M., Gagnor, C., Milhaud, P.G., Imbach, J.-L. and Lebleu, B. (1988) Gene 72, 323–332.
[22] Kabanov, A.V., Chikindas, M.L., Astafyeva, I.N., Glukhov, A.I., Gordeev, S.A., Izumrudov, V.A., Zezin, A.B., Levashov, A.V., Severin, E.S. and Kabanov, V.A. (1989) Dokl. Akad. Nauk SSSR 306, 226–229.