In Vivo Oligonucleotide-Protein Interactions in the Blood

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The interaction of oligonucleotides with serum proteins was studied *in vivo* by the method of affinity modification. Alkylating oligonucleotide derivatives administered to animals induced the formation of oligonucleotide-protein complexes circulating in the blood for a long-time. Oligonucleotides in these complexes were partially protected from nucleases. The major oligonucleotide-binding proteins identified with specific antibodies were albumins and IgG. In the erythrocyte fraction no specific oligonucleotide-binding proteins were detected, while membrane-cytosolic leukocyte fraction contained an oligonucleotide-binding protein with a molecular weight of 72 kD.

Key Words: *oligonucleotides; affinity modification; serum proteins; immunoglobulins; albumins*

Oligonucleotides (ON) are successfully used for inhibition of virus replication and regulation of oncogene expression and can be used for creation of antiviral and antitumor drugs [10]. The study of metabolism and interaction of ON with biopolymers in living organisms is important for evaluation of possible adverse effects and improvement of their pharmacological properties. ON are distributed and redistributed in organs and tissues through the blood and their lifespan depends on nucleotide-nucleotide bonds and the presence of modified terminal groups in their secondary structure [5]. Binding of ON with serum protein was demonstrated *in vitro* [2].

In the present study we investigated the interaction of ON derivatives carrying 5'-terminal alkylating group (4-[(N-2-chloroethyl-N-methyl)amino] benzylamine with serum proteins after intraperitoneal and peroral administration to BALB/c mice.

MATERIALS AND METHODS

Deoxyribooligonucleotides pTGACCCTCTTCCCATT — $p(N)_{16}$ and $pT(TC)_6T$ — $p(N)_{14}$ were synthesized by phosphotriester method in solution [3]. The radioactive label ^{32}P was introduced into ON by transferring the terminal phosphate from γ-^{32}P-ATP (BIOSAN, specific activity 5×10^3 Ci/mmol) to the 5' position of ON using T4 polynucleotide kinase (Sibenzim). ON were purified from reaction products by electrophoresis (20% polyacrylamide gel: $^1/_{20}$ bis-acrylamide, 8 M urea, 0.5 M EDTA, 50 mM Tris-borate buffer, pH 8.3), transferred to DE-52-cellulose (Whatman), and eluted from cellulose with 2 M $LiClO_4$ in acetone (centrifugation at 14,000 rpm for 5 min in an Eppendorf 5415 C centrifuge). The pellet was washed with acetone, dried, dissolved in H_2O, and the concentration of ON was determined spectrophotometrically. Molar coefficients were calculated as described previously [8] on the basis of known ε=260 for mono- and dinucleotides [9]. Specific radioactivity of synthesized ON was 25-100 Ci/mmol.

Alkylating group was introduced by binding (4-[(N-2-chloroethyl-N-methyl)amino]benzylamine to 5'-terminal phosphate of ON [1]. The yield of ON derivatives and the presence of active chlorine in alkylating group were determined by the reaction with 0.5 M sodium thiosulfate followed by analysis of reaction products by electrophoresis in 20% polyacrylamide gel ($^1/_{20}$ bis-acrylamide) containing 8 M urea, 1 M EDTA, and 50 mM Tris-borate buffer (pH 8.4). The yield of alkylating ON derivatives was no less than 90%.

Modified ON were administered to BALB/c mice weighing 25-27 g in a dose of 0.5 mg/kg in 200 μl

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physiological saline (intraperitoneal injection) or 25 μl water (*per os*).

Blood was collected from the retroorbital sinus 20 and 40 min and 1.5, 3, 9, and 24 h after administration of ON. Serum was obtained from 20-30 μl blood after 40-min incubation at room temperature and centrifugation. Serum proteins affinity modified by alkylating ON derivative were analyzed by disk-electrophoresis in 10-20% polyacrylamide gradient gel as described previously [11].

Analysis of ON incorporated into ON-protein complexes was carried out after hydrolysis of phosphamide bond between the reactive group and 5'-terminal phosphate of ON. Fragments of polyacrylamide gel containing modified serum proteins were treated with 5% perchloric acid for 3 h at 10°C. The extract was neutralized with 5% LiOH, filtered, and ON were separated by electrophoresis followed by autoradiography.

Immunochemical identification of ON-binding proteins was performed by the immunodiffusion method. Antibodies against all mouse serum proteins and affinity purified anti-mouse IgG antibodies were used as secondary antibodies.

Blood cells were separated into erythrocytes and serum fraction by centrifugation in a density gradient [7]. Membrane-cytosolic fraction was prepared by treating the cells with 1% NP-40 [13]. ON-binding proteins were analyzed by electrophoresis in polyacrylamide gel as described for serum proteins.

RESULTS

ON can interact *in vitro* with serum albumin, IgG, and IgM [14]. *In vivo* ON-protein interactions were studied using affinity modification of serum proteins with alkylating ON derivatives. Deoxyribooligonucleotides $p(N)_{16}$ and $p(N)_{14}$ carrying (4-[(N-2-chloroethyl-N-methyl)amino]benzylamine at the 5'-terminal phosphate ($ClRp(N)_{16}$ and $ClRp(N)_{14}$) were used as affinity agents. After intraperitoneal administration of the alkylating ON derivative to mice, blood was collected, separated into cellular and serum fractions, and analyzed by SDS-disk-electrophoresis in polyacrylamide gel.

In the serum fraction, 2 proteins affinity-modified by alkylating ON derivative and corresponding to albumin and IgG heavy chain were found (Fig. 1). Experimental data suggest that ON-protein complexes appeared in the blood immediately after injection of ON and persisted in the organism for a long-time. Their concentration peaked 3-9 h postinjection. Peroral administration yielded a far lower concentration of ON-protein complexes probably due to their less effective penetration (data not shown).

Serum proteins forming complexes with ON were identified by immunodiffusion of the serum obtained 6 h after intraperitoneal injection of $ClRp(N)_{16}$ against antiserum to mouse serum proteins and affinity-purified anti-mouse IgG antibodies followed by autoradiography (Fig. 2). The presence of a precipitate with anti-mouse

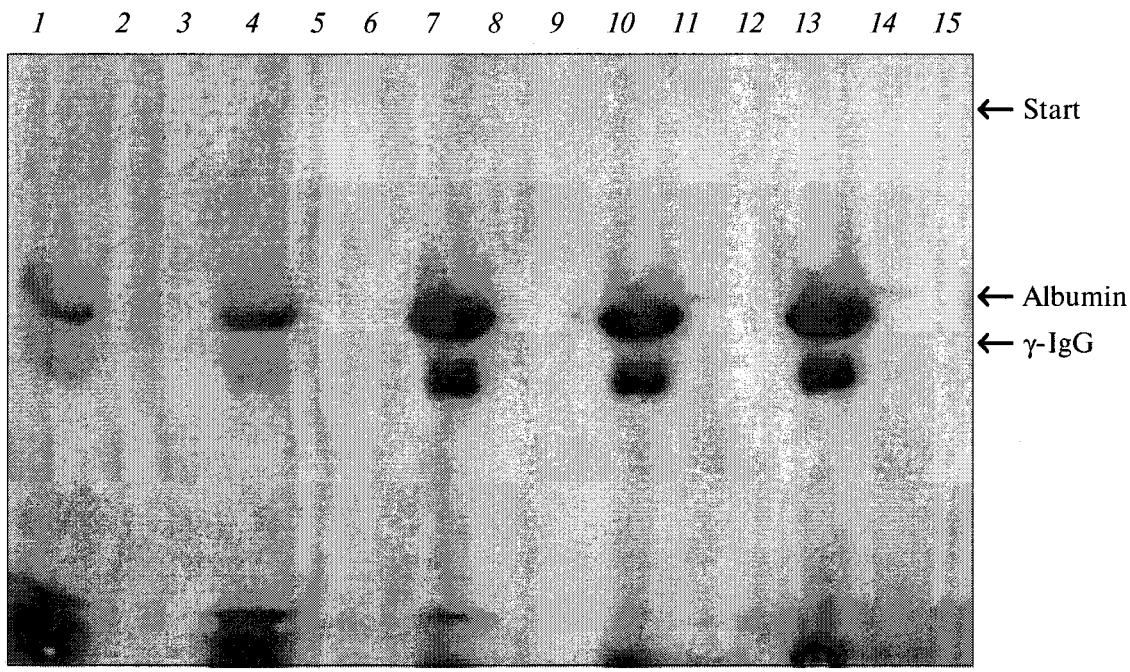

Fig. 1. Modification of serum proteins and blood cell elements in BALB/c mice after intraperitoneal injection of 0.5 mg/kg ^{32}P-$ClRp(N)_{16}$. SDS-disk-electrophoresis in 10-20% polyacrylamide gradient gel, autoradiograph. Runs *1, 4, 7, 10,* and *13* correspond to serum proteins, runs *2, 5, 8, 11,* and *14* to leukocytic fraction, and runs *3, 6, 9, 12,* and *15* to erythrocyte fraction after 0.66, 1.5, 3, 9. and 24 h, respectively.

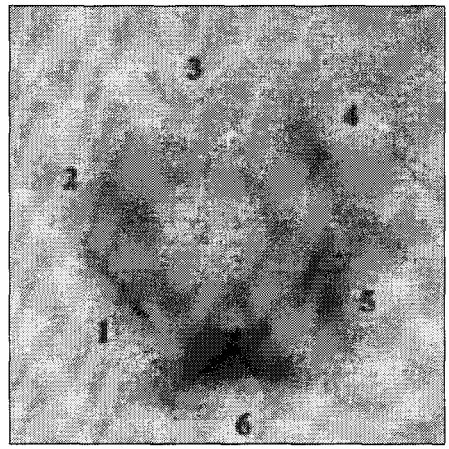

Fig. 2. Immunodiffusion of mouse serum after intraperitoneal injection of 0.5 mg/kg ^{32}P-ClRp(N)$_{16}$ followed by autoradiography. *1-3)* goat anti-mouse IgG antibodies (0.1 mg/ml) diluted $^1/_2$, $^1/_4$, and $^1/_8$, respectively; *4)* goat antibodies to mouse albumin (0.01 mg/ml), *5,6)* goat antiserum to mouse serum proteins diluted $^1/_2$ and $^1/_4$, respectively.

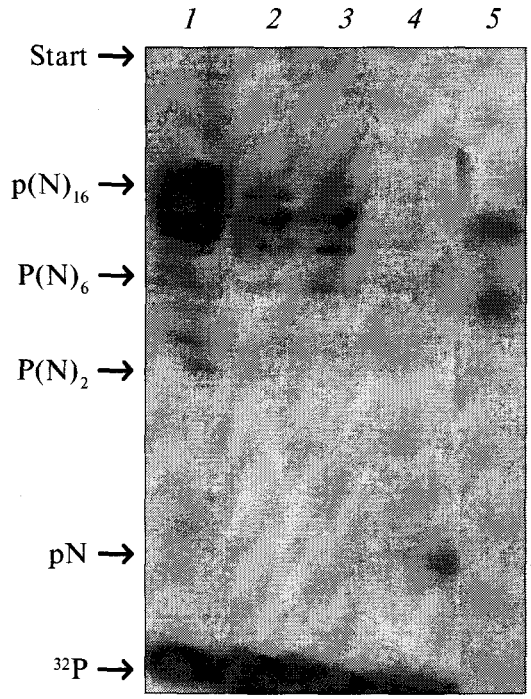

Fig. 3. Integrity of ^{32}P-ClRp(N)$_{16}$ in mouse serum after intraperitoneal injection. Electrophoresis in 20% polyacrylamide ($^1/_{20}$ bis-acrylamide), 8 M urea, autoradiograph. *1)* marker oligonucleotides; *2-4)* serum oligonucleotides 0.3, 0.7, and 1.5 h after injection of ClRp(N)$_{16}$, respectively; *5)* oligonucleotides associated with serum proteins, 24 h after injection of ClRp(N)$_{16}$.

IgG antibodies suggests that affinity-modified proteins include mouse IgG, while the presence of a characteristic precipitate with antibodies to mouse serum proteins suggest affinity modification of albumin (Fig. 2).

In our previous *in vitro* experiments we showed that ON bind to Fab fragment of Ig and do not affect its antigen-binding capacity, whereas Ig in immune complexes do not interact with ON. The binding is primarily effected through the formation of ionic bonds and can be inhibited by non-nucleotide polyanions, i.e., is nonspecific [4]. *In vivo* formation of ON-Ig complexes is most probably underlain by the same mechanism (binding with anion-binding site) and can modulate the effector properties of Ig, in particular receptor binding.

For evaluation of the effect of complexation with proteins on the lifespan of ON in the circulation, the total fraction of ON-binding proteins was isolated from the serum obtained 24 h after administration of ON by elution from polyacrylamide gel after SDS-disk-electrophoresis. The acid-sensitive bonds were cleaved with 5% $HClO_4$ and free ON were analyzed by electrophoresis in 20% polyacrylamide gel (Fig. 3). It was shown that the complexes contained ON consisting of 4-5 and 7-8 nucleotides. Since phosphodiester bonds in ON are rapidly cleaved by serum nucleases, the high stability of ON incorporated into ON-protein complexes is apparently due to shielding, which preventing nuclease binding, thus prolongs ON lifespan, and promotes their distribution in organs and tissues. It should be noted that 40 min after administration of ClRp(N)$_{16}$ to mice, no fragments of the initial ON (except for 6- and 8-nucleotide fragment) were detected (Fig. 3). In this experiment, serum samples were not treated with 5% $HClO_4$ (which led to hydrolysis of phosphoamide bonds and the release of ON from the ON-protein covalent complexes), hence, the formation of noncovalent ON-protein complexes also protects ON from nucleases.

In the membrane-cytosolic fraction of erythrocytes, no proteins forming complexes with ON at the studied concentrations were detected. In the membrane-cytosolic fraction of leukocytes an ON-binding protein with a molecular weight of 72 kD was found. This protein is similar to that discovered in K-562 cells [6] and splenocytes [12] and probably belongs to Ig receptor family [12].

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