INTRODUCTION

Oligonucleotides regulate gene expression by several mechanisms: antisense interactions [1, 2], RNA internalization [3, 4], and aptamer effects on regulatory proteins [5–8].

Oligonucleotides can be transferred into the cell by receptor-mediated endocytosis, which involves specific receptors [9–11]. The role of the interaction with the cell membrane in specific functions of oligonucleotides is still poorly understood. We previously studied the effect of low concentrations of antisense oligodeoxynucleotides (dONs) on transcription of the BCR/ABL oncogene and observed modulation of the transcriptional activity for some other genes, but not for BCR/ABL [12]. This finding makes it possible to assume that specific cell processes are triggered by dONs, which interact first with cell receptors to enter the cell.

To determine whether the highly specific binding and internalization of dON changes expression of a particular gene, we estimated the parameters of the interaction with K562 cells for an octadecameric dON containing the translation start of the BCL2 mRNA. Cells of the K562 line express BCR/ABL, which determines cell resistance to cytostatic agents and lowers sensitivity to growth factors in combination with mutant p53. Owing to these properties, K562 cells provide a convenient model for studying the interaction of cancer cells with oligonucleotides.

We estimated the binding constant, the number of binding sites on the cell membrane, and the extent of internalization for dON under standard conditions and upon lipofection. Internalization proved to follow high-affinity receptor-mediated binding of dON. This process was accompanied by changes in the rate and extent of internalization and with priming of new high-affinity binding sites.

EXPERIMENTAL

Oligonucleotides. Tritiated dON 5'-AAGGATG-GCGCACGCTG-r[3H]A-3' was obtained by 3'-end labeling of 17-mer dON containing the BCL2 mRNA translation start (2.3 nmol) with 0.3 mCi of [2.8-3H]rATP (29 Ci/mmol, Nuklon, Russia) and 100 units of terminal deoxynucleotidyl transferase in 0.2 ml of reaction buffer (Promega, United States) at 37°C for 16 h [13]. Gel filtration on Sephadex G-25 (14 ml) was used to purify dON from rATP. The specific radioactivity of dON was 42.9 Ci/mmol, and the binding was 1.3 [H]A per mol dON.
The heptadecamer to be labeled and carboxyfluorescein-labeled dON (5'-FAM-dON) were synthesized by Sintol (Moscow) and purified by gel electrophoresis.

**K562 cells** (BCR/ALB*, wt-p53, b3a2 mRNA BCR/ABL) were obtained from the collection of the Institute of Cytology (St. Petersburg). Cells (6–10 · 10⁵/ml) were cultured for 3 days in RPMI 1640 supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 10% fetal calf serum (FCS) in 25-ml plastic flasks (Flow Laboratories, United Kingdom) in an atmosphere containing 5% CO₂ at 37°C. Then cells were washed with RPMI 1640 to remove FCS and collected by centrifugation at 450 g at room temperature.

**Binding of dON with K562 cells.** Cells were prepared as above, without FCS. Binding was assayed in RPMI 1640 containing 29 nM dON and 10⁶ cells/ml in an atmosphere containing 5% CO₂ at 37°C for 1, 2, or 4 h, separately for each sample. Cells were collected by centrifugation at 450 g at 10°C for 10 min, washed with PBS (0.15 M NaCl, 9 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4, 10°C) at 10⁶ cells/ml for 30 min (the time it takes for an equilibrium to be achieved), and centrifuged again. Experiments were repeated twice in total volumes of 1 and 7 ml. Cell mortality was 10% upon transfer into a FCS-free medium (time 0) and increased by 5–7% during 6 h incubation in an atmosphere containing 5% CO₂ at 37°C.

The total (dON₂) and surface (dONₐ) binding with K562 cells (pmol per 10⁶ cells) and the extent of dON internalization were assayed by scintillation counting in a toluene–Triton solution on a Beckman counter.

The total binding was measured by [³H]dON in an alkaline lysate of 2 · 10⁶ cells in 1 ml of 5 M KOH (5 min, 60°C) [14].

The surface binding was assayed in the supernatant of 10⁶ dON–K562 complexes treated with 1 ml of an acid–salt solution (ASS; 0.2 M CH₃COOH, 0.5 M NaCl, pH 2.5) [15] at no more than 10°C. After extraction of dONₐ with ASS, the extent of internalization (dONₖ) was assayed in an alkaline lysate of the cell pellet.

**Interaction of K562 cells with dON contained in liposomes.** was studied as above, in FCS-free RPMI 1640 containing 10⁶ cells/ml and 29 nM dON. To obtain a dON–liposome conjugate in deionized water with a 7 : 1 ratio between the (+) charge of liposomes and (–) charge of dON, 19 µl of 1 mM bicationic lipid Tfx-50 were mixed with 300 pmol of dON for 40 min at room temperature. To obtain the lipid preparation, 0.7 mg of a mixture of bicationic and neutral lipids and 400 µl of nuclease-free water were shaken for 10 s at room temperature, incubated for 1 min at 65°C, and stored at −20°C as recommended by Promega. The conjugate was used at 20 µl per ml cell suspension (10⁶ cells/ml). The resulting cell suspension contained 29 nM dON conjugated with liposomes. Aliquots of the suspension were incubated at 37°C for 1, 2, or 4 h in duplicate. The dON–K562 complexes were collected by centrifugation at 10°C and used to estimate dON₂, dONₐ, and dONₖ as described above.

**Binding constant Kₘ and binding site number B₀** were estimated by stepwise dissociation of dON–K562 cells in PBS under the equilibrium conditions with further internalization being stopped [16]. Bound (dONₗₐ) and free (dONₗₐ) dON and their ratio were determined; the results were presented as Scatchard plots to estimate Kₘ and B₀. For this purpose, 10⁶ dON–K562 cells were incubated in 1 ml of PBS at 10°C for 10 min and centrifuged at 4°C at 450 g for 20 min, and [dON]ₗ(1) was measured in the supernatant. Pellet (1) was treated with ASS as described for the supernatant, centrifuged, and used to estimate [dON]ₗ(2). After the first round of equilibrium dissociation, three or four additional rounds were carried out with PBS similarly. Each round ended with centrifugation and measurement of [dON]ₗ(2), [dON]ₗ(3), [dON]ₗ(4), etc., in the supernatant. Equilibrium concentrations of dON bound on the cell surface ([dON]ₗ(2), [dON]ₗ(3), and [dON]ₗ(4)) were measured in the ASS extracts of the corresponding pellets obtained by centrifuging each sample in PBS. Stepwise dissociation was carried out in duplicate, and [dON]ₗ(1) was determined for all six complexes, including those obtained by lipofection. The results were not corrected for reverse hydrogen–tritium exchange, which was ≤0.014‰ under the conditions used [17]. The linearity of Scatchard plots testified that equilibrium was achieved at every dissociation step.

**Kinetics of the total FAM-dON binding with K562 cells** was studied as described for [³H]dON in the absence of FCS. FAM-dON was used at 29 nM. The culture medium was removed by centrifuging samples at 450 g at room temperature for 8 min; 10⁶ cells were resuspended in 0.5 ml of FCS-free RPMI 1640 and stored at 4°C for no more than 3 h. The FAM-dON content was inferred from cell fluorescence, which was measured by flow cytofluorometry on an EPICS-XL instrument (Beckman Coulter, United States) with 10⁴ cells at 200–500 cells/s. The fluorescence of cells accepting FAM-dON was estimated from the mean peak value on frequency histograms.

**RESULTS**

To study the interaction of K562 cells with the tritiated dON 5'-AAGGATGGCGCACGCTG-[³H]rA-3', we characterized the kinetics of accumulation of total (dON₂), surface (dONₐ), and intracellular (internalized, dONₖ) at 37°C (Figs. 1, 2) and estimated the binding constant and the number of binding sites at 10°C, under conditions preventing internalization [14] (Figs. 3, 4).