IL-13 GENE TRANSLATION IS ARRESTED BY A NOVEL OLIGONUCLEOTIDE IN CULTURED HUMAN B- LYMPHOCYTES

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ABSTRACT

Antisense oligodeoxynucleotides (oligos) are the tools that bind to complementary sequence of targeted mRNA and block specifically protein translation. In the present study, a novel 20 mer oligo as an antisense for human IL-13 is introduced. This oligo is designed according to the IL-13 mRNA coding region and synthesized in two HPLC purified and FITC conjugated forms. Fluorescence oligo cell uptake is confirmed using flowcytometry and confocal microscopy, and cytotoxicity evaluation is performed using BrdU proliferation assay. Human tonsilar B-lymphocytes are purified by positive selection using magnetic cell sorting method and cultured with anti CD40 monoclonal antibody plus rIL-4 to induce IL-13 production. IL-13 antisense is added to medium and Real Time PCR for mRNA, and ELISA for protein assays. Data indicate that antisense application leads to down regulation and complete suppression of IL-13 protein with no significant effects on mRNA, suggesting in vitro protein translation arrest. Since Il-13 is a crucial cytokine in allergic conditions, we conclude that interference with the protein synthesis by a nontoxic and efficient antisense oligo can provide an available tool for the investigators on allergic diseases.

Keywords: Antisense, Allergy, Human IL-13.

INTRODUCTION

Antisense oligodeoxynucleotides (oligos) are designed to switch off gene expression by interfering specifically with the translation of the encoded protein at the mRNA level.[1] Zamecnik and Stephenson originally introduced the exciting concept of using antisense oligos to block expression of a single gene in 1987.[2,3] By definition, the m-RNA is the "sense" strand, and any complementary sequence is " Antisense" to it. So the oligo hybridizes to that part of the m-RNA that contains the complementary sequence and the cell becomes deficient only of the specific gene while other portions are regularly transcribed.[4] Generally any sequenced gene may be targeted by Antisense oligos and even highly homologous members of a gene family may be inhibited selectively.[5] A large number of investigators targeted lymphocytes derived cytokines by Antisense oligos,[6,7,8] but there is no report on human IL-13 inhibition so far. IL-13 is a B-cell stimulatory cytokine, which is a crucial contributor to the pathogenesis of asthma and anaphylaxis.[9] This strong connection to allergic diseases makes IL-13 a potential therapeutic target for atopic diseases.[10] In the present study, we tried first to set up a specific cell culture condition in order to direct to IL-13 production. Ac

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cording to previous reports,[11] B-cells can secrete IL-13 under certain stimuli such as anti CD40 monoclonal antibody, and recombinant IL-4. Blocking the IL-13 secretion is required in studies on biological effects of this cytokine and it's importance in allergic patients. Our experiment is based on previous works, which have been conducted in Meakins-Christie Laboratory to determine autocrine effects of IL-13 on human B-cells.[12] However it has already examined several blockers such as anti IL-13 and anti receptor and monoclonal antibodies, with partial, but not totally successful on IL-13 inhibition.[13] However, antisense technique used in this study is a new approach to get complete IL-13 blocking and apply in further researches on allergic diseases.

MATERIALS AND METHODS

Design and Synthesis of IL-13 Antisense

Using aopredict program on www. Cgb ki. Sc. web site, the human IL-13 antisense 20- mer oligo was designed according to entire m-RNA sequence targeted to coding region (442 bp from 15-457) in two forms, HPLC purified phosphothioate, and FITC conjugated. At first all the sequences were compared with each other and with database which contained the complete molecular characteristics of all tested antisense ODNs. The sequences that showed relevant cross homologies to other gene bank sequences were excluded. Finally, all remaining candidate sequences were ranked using the blast program, and the top sequence which was complementary to nucleotide 366-385 of IL-13 m-RNA, 5'-CAA ACT GGG CCT CGA TT-3 was selected and synthesized by invitrogen company.

Preparation of tonsillar B-cells

mononuclear cells are first isolated from human tonsils using ficol-paque density gradient centrifugation.[12] B-cells are purified by positive selection using magnetic cell sorting method (MACS). In this method colloidal super-paramagnetic micro beads conjugated to mouse monoclonal CD19 antibody are used according to supplier procedure (Miltonvi Biotec, USA). Cells are magnetically labeled with CD19 micro beads and separated on a column, which is placed in the magnetic field. The magnetically labeled cells are retained in the column and can be eluted as positively selected cell fraction.

Cell culture

In order to direct purified B-cells to produce IL-13, we cultured lymphocytes in complete medium (RPMI 1640, 10% FBS, 4 mM glutamine, 100 U/mL penicilin. G, 100 μg/mL streptomycin, 1 mM MEM sodium pyruvate, and 15 mM HEPES buffer) in 24 wells cell culture micro plate, and stimulated by adding monoclonal antibody to CD-40 (1 μg/mL), plus recombinant human IL-4 (400U/ml). The IL-13 antisense oligo was added (2 μM) into separate wells at the beginning and day 3 of the culture. In order to evaluate the inhibitory effects of IL-13 antisense, after collecting the supernatant in day 3, cells were suspended in complete medium plus oligo and cultured up to 14 days without stimulatory reagents (anti CD40 and rIL-4). The supernatants were collected at days 1,5,7,10, and 14, during 14 days cell culture and kept at –20 until used.

Flowcytometry

For oligo cell uptake analysis flourescence labeled oligo was added to B-cell culture media (2μM) supplemented with anti CD40 antibody plus recombinant human interleukine-4 (20,000 cell/well in 24 wells micro plate). After given hours (2, 4, 8, 18, 24 and 48 hours), cells were collected, washed in PBS-BSA 1%, and samples analyzed using a FACScan flow cytometer (Becton Dickinson, USA), for flourescence positive cell numbers.

Confocal Microscopy

To prepare microscopic slides, purified tonsillar B-cells were plated in pre-washed 96 wells cell culture micro plate in complete medium plus anti CD-40, rIL-4 and 2μM oligo (3000 cells/150 μL). The incubations were stopped at 48, 24, 8, 4, 2 and 1 hours time points by transferring cells into microfuge tubes. Cells were spun at 400g and washed twice with minimal medium. Cells were re-suspended and attached to a poly L-lysine pre-coated slide by cytocentrifugation. Slides were air dried at 37°C and fixed in 100% methanol (-20°C) for 5 minutes, dehydrated in a graded series of alcohol (70%-100%) and fully air-dried, then were evaluated under the fluorescence and confocal microscopes after adding nonquenching immersion oil.

BrdU uptake assay

In order to evaluate the toxicity effects of antisense oligo, tonsillar B-cells were isolated and cultured in 24 wells micro plate (1×10^6 cells/ mL) in the presence of anti CD-40 monoclonal antibody plus recombinant human IL-4. Antisense oligo was also added to separate wells at the same time (2 nMol / ml). BrdU flow kit (BD company, USA) was applied to determine the number of proliferative cells in the presence and absence of oligo. To label the cells in vitro, BrdU solution (1mM) was carefully added directly to tissue culture media. After 1-hour incubation at 37°C, cells were harvested, fixed, permabilized, and stained for incorporated BrdU using fluorescent anti-BrdU antibody and 7 ADD for DNA contents. Cells were analyzed by flowcytometer for FL-1 (green color), and FL-3 (red color) detectors to determine the proliferation rate and cell cycle DNA contents respectively.

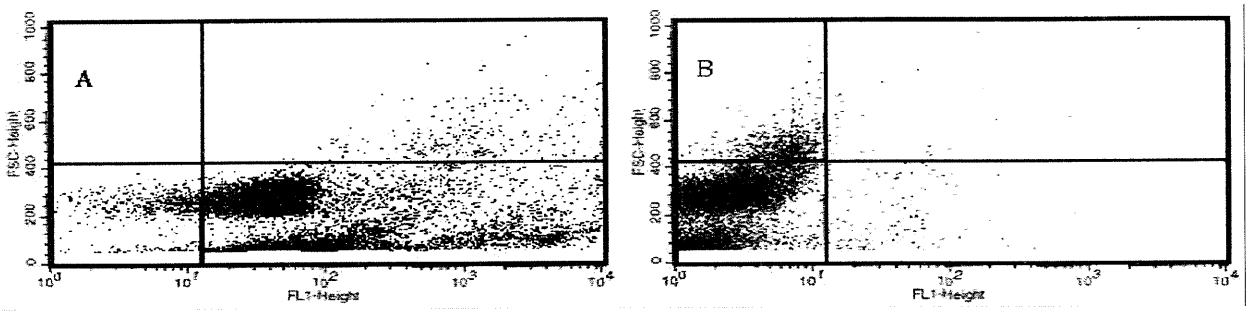

Fig. 1. Fluorescein conjugated oligo uptake after 1h cell culture. Incubation of B-cells with labeled oligo leads to FL1 positive cells (A) in comparison with negative control (B).

Tripan blue exclusion assay

The lymphocyte cytotoxicity was also measured by tripan blue exclusion method using 0.5% w/v tripan blue / 0.9% NaCl. Cells were plated in 24-wells flat bottom micro titer plates at 50-70% confluency in supplemented medium. The identical plates were prepared for time points. Specific antisense oligo was added after 4 hours in increasing amounts to 4 replicate wells (0.5, 2, 5, 10, 20, 50 μM). Cells were incubated for 24h, collected and washed once in PBS to stop incubation, and 600μL of 0.5% tripan blue was added to each well. The cell number and percentage of dead cells were determined under the microscope using a hemocytometer.

Real time PCR

Tonsillar B-cells were cultured in complete medium in the presence of anti CD-40, rIL-4 and oligo (2μM). IL-13 determination was carried out in the level of m-RNA by real time PCR after 24 and 36 hours. In order to extract and quantitate m-RNA, harvested cells were first treated by trizol reagent (GIBCO BRL) according to supplier's procedure. Then RNA reverse transcription reaction was carried out using RT mix solution (first strand buffer, DTT 10 mM, dNTP 1mM, RNase inhibitor 20U, and MML-V RT enzyme 200U), after incubating 1μg extracted RNA with 0.5μg oligo dT primers (invitrogen), and denaturing RNA and primers for 10 minutes at 65°C. RT- PCR Products were applied in real-time reaction using light cycler-primer set (human interleukin-13) which was ready to use amplification primer mix for RT-PCR using the light cycler instrument (Gmb Heidelberg).

ELISA

Cell culture supernatants were harvested on days 1,5,7,10, and 14, and IL-13 protein was measured by ELISA using IL-13 Eli-Pair kit (DIACLONE, UK) according to the company's procedure.

Statistics

Normally distributed data are expressed as mean ± SEM and assessed for significance by student's t-test or ANOVA as appropriate. Data that were not normally distributed were assessed for significance using the Wilcoxon rank sum test.

RESULTS

Evaluations of ODN cell incorporation

Flowcytometric, fluorescence and confocal microscopic evaluations were taken placed within 1-72 hours after cell culture in comparison with negative controls (without oligo). Flowcytometric results revealed that spontaneous cellular uptake of oligo without helping reagents is above 90% after 1 hr (Fig 1) and sustains for 72 hrs follow up. Fluorescence and confocal microscopic results after 4 hours incubation are also shown in Figs 2, 3.

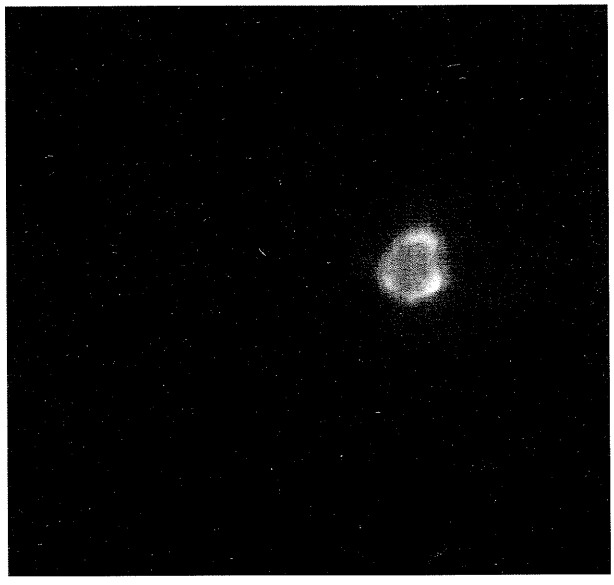

Fig. 2. Fluorescent microscopic picture of fluorescein labeled antisense incorporation into cultured B-cells.

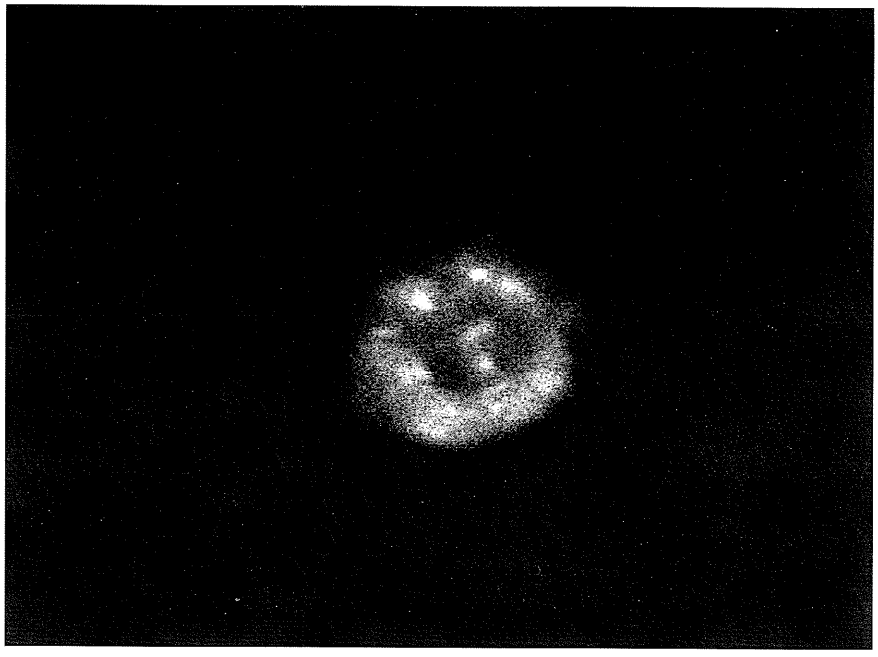

Fig. 3. confocal microscopic picture of fluorescence oligo incorporation in B-cell. Punctuate intracellular distribution is caused by entrapment of the oligo in endosomes from where the oligos are slowly released to other cytoplasmatic compartments.

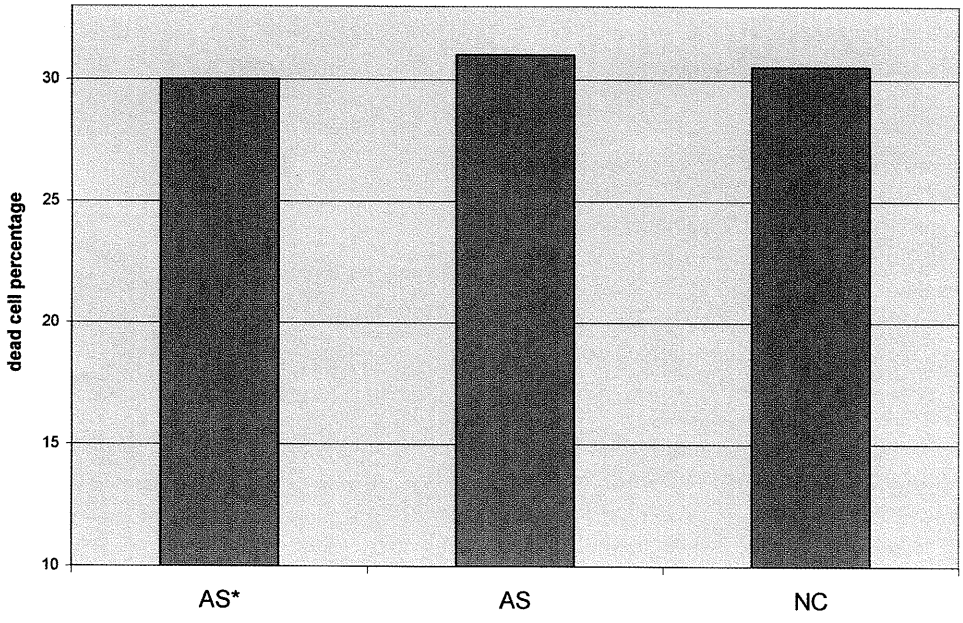

Fig. 4. Representative values for tripan blue exclusion assay. Once addition of oligo on day 0 (AS), twice addition on days 0 and 3 (AS*), and no addition of oligo (NC) had no significant difference at the numbers of mean percentage for 6 increasing amounts of oligo (0.5, 2, 5, 10, 20, 50 μ M).

Antisense cytotoxicity assay

Cell viability assay in the presence of IL-13 antisense oligo, in comparison with negative control, using tripan blue staining (represented in Fig 4), showed no significant differences in dead cell number (31% and 30.5% mean values respectively). Moreover, oligo addition to culture medium on day 3 had no significant effect(s) on dead cell number (30%).

Cytotoxicity evaluation of IL-13 antisense using BrdU test indicated no toxic effects on cell growth and

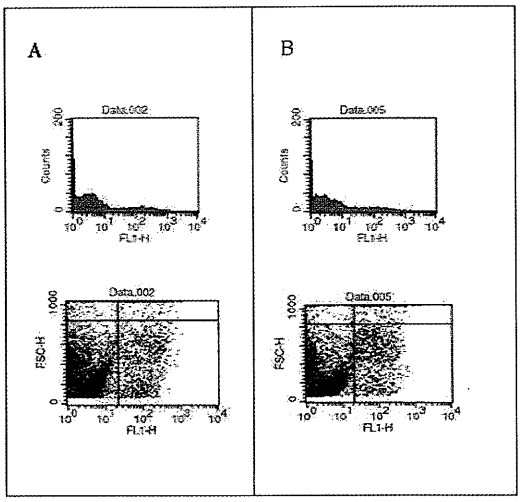

Fig. 5. Cell proliferation assay with BrdU uptake. The number of proliferative cells in test sample with Antisense oligo (A), and negative control, without oligo (B) have no significant difference indicating no toxic effect of oligo on cultured cells.

the percentage of proliferative cells after 5 days cell culture in the presence of IL-13 antisense in comparison with negative control (10.7% and 11.3% proliferative cells respectively). These results are presented in Fig 5.

Antisense inhibitory activity on IL-13 production

Real time PCR determined no significant differences between IL-13 m-RNA contents of tonsillar B-cells incubated with IL-13 Antisense compared with negative control. But ELISA IL-13 protein assay, after 5, 7, 10 and 14 days culture, revealed that the amounts of this cytokine in comparison with negative control had 71%, 70%, 82% and 67% reduction , respectively. In another experiment when antisense was added again on day 3, more reductions were demonstrated in IL-13 concentrations (100%, 84%, 79%, 66% respectively), and resuspending the cells in fresh medium without anti CD40 antibody and rIL-4 led to totall inhibition in IL-13 secretion in the cells previously affected by Antisense oligo (Fig 6).

DISCUSSION

Antisense oligonucleotides have proven to be effective and highly specific inhibitors of gene expression. Increasing knowledge about the biological role of genes makes the Antisense technique a rational approach to study gene function and for drug development [1,5]. The nucleotide composition, the secondary structures of the target m-RNA and hybridization kinetics are important criteria for ODN designing. An oligo design should lead to the ability to cause a highly specific and efficient translation block.[15]

In the present study, we designed a novel 20 mer Antisense oligo in accordance with previously reported human IL-13 m-RNA sequence.[16] The Antisense was applied to down regulate translation of IL-13 in order to investigate its influence(s) on the other cell factors mainly in allergic and asthmatic patients, using previous reported data on antisense technology and its applications for down regulation of other genes.[17,18] In

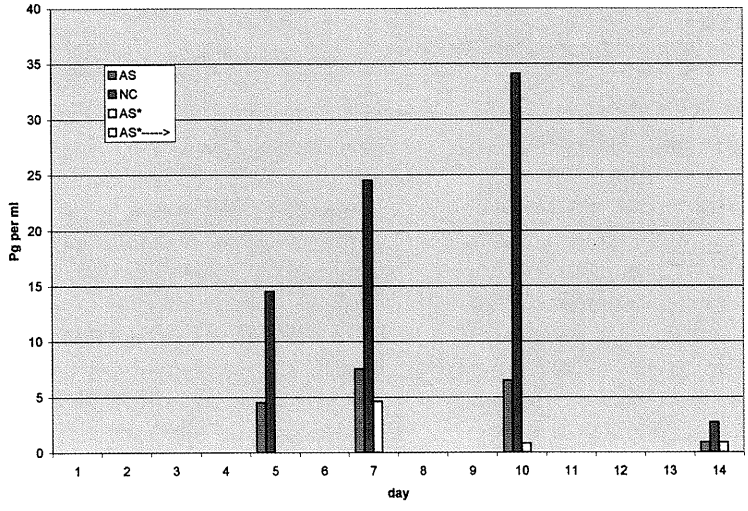

Fig. 6. IL-13 concentration in cell culture supernatants. Protein assay during 14 days cell culture indicated significant decrease after Antisense oligo application on day 0 (AS), higher decrease with twice oligo addition on days 0 and 3 (AS*), and complete suppression after removing anti CD40 and rIL-4 on day 3 (AS*—>) in comparison with no addition of oligo (NC).

Table I. IL-13 m-RNA and protein changes in different cell culture conditions. NS=Not Seen.

Cell Culture	Anti CD40 & r-IL4 addition	Antisense addition 2μM/ml	m-RNA changes	% IL-13 protein reduction			
				Day 5	Day 7	Day 10	Day 14
1 million B-Lymphocytes / Complete Medium	Day 0	Once, day 0	N.S.	71	70	82	67
	Day 0	Twice, days 0 & 3	N.S.	100	84	79	66
	Day 0, removal on day 3	Twice, days 0 & 3	N.S.	100	100	100	100

order to orient cultured cells to IL-13 secretion and to eliminate other cytokine cross reactivity, T cells and monocytes were depleted from mononuclear population. Therefore, purification of B-lymphocytes using specific magnetic beads was applied to eliminate several T-cell derived cytokines. Moreover tripan blue staining and BrdU cell uptake experiments revealed no evidence of antisense cytotoxicity effects on cell growth and proliferation. This has been attributed to chemical toxicity removal via post synthesis reversed phase HPLC purification of oligo, and a decreased non-specific binding to related or non related RNA sequences, by sequence specific selection oligo for IL-13 mRNA coding region. High efficiency transfection of Antisense confirmed by flowcytometry and confocal microscopy indicated that HPLC purified oligo code incorporate into cultured cells with no need for helping reagents.

It has been suggested that following the ligation of CD40, and addition of cytokine, human B-cells demonstrate significant increase in IL-13 m-RNA.[17] We cultured purified human tonsillar B-lymphocytes in the presence of anti CD40 monoclonal antibody as a T-B cell interaction substitute, plus rIL-4 to promote IL-13 secretion. Moreover oligo application schedule including its dosage and time course addition was set up in our laboratory. Data represented in Table I indicate that although a single application of oligo can reduce IL-13 secretion, but twice addition on days 0 and 3 is more efficient on IL-13 gene down regulation. However it was observed that cell culture medium replacement with fresh complete medium without anti CD40 and IL-4 on day 3, does not induce stimulation of new IL-13 producing clones, and provides only the survival conditions for previously primed B cells. We suggest that these primed cells are the cells influenced by Antisense oligo and in this condition IL-13 secretion is completely blocked suggesting its perfect blocking effect on protein translation process.

Data reported in this study indicate that this Antisense oligo acts permanently on ribosome and m-RNA level to interfere with protein translation. Since no m-RNA decrease was observed in real time testing, we concluded that in contrast to tissues of very high proliferative activity like emberyonic tissue, Rnase H had no significant role in m-RNA cleavage and down regulation of protein translation of B- lymphocytes. As expected,[18] the antisense designed according to m-RNA coding region, had permanent inhibitory effect on post transcription phase of IL-13 protein. Therefore it is not able to cleavage IL-13 m RNA after hybridization with Antisense oligo.

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