Inhibition of IL-13 by Antisense Oligonucleotide Changes Immunoglobulin Isotype Profile in Cultured B-Lymphocytes

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ABSTRACT

The link between IL-13 and bronchial hyper-responsiveness has brought this cytokine as a potential therapeutic target for asthma and allergic diseases. At the present study, we address the role of B cell derived IL-13 in the IgE and other immunoglobulin development. Antisense oligo for human IL-13 m-RNA was used to study IgE down regulation. Human B-lymphocytes were purified by positive selection using magnetic cell sorting and were cultured in the complete medium plus anti-CD40 monoclonal antibody and recombinant human IL-4. Immunoglobulin assay was performed by ELISA in the presence and absence of antisense oligonucleotide. We demonstrated that IL-13 antisense causes the decrease of IgE and increase of IgA significantly and no significant changes in IgM and IgG levels ($p < 0.01$). We also demonstrated that both IL-13 inhibition and IL-4 removal cause the complete blocking of IgE and significant decrease of IgM and IgG levels. Our IL-13 antisense oligo can block B-cell IL-13 productions and consequently inhibits IgE production followed by IgA class switching *in vitro*. We suggest that in contrast to the IL-4, IL-13 is apparently more potent in the IgE switching and has no significant role in IgG and IgM levels. *Iran. Biomed. J. 8 (4): 185-191, 2004*

INTRODUCTION

Antisense oligonucleotides (ODN) are tools designed to bind specifically and efficiently to the complementary sequence of targeted m-RNA, that lead to specific and efficient translation block. ODN are actively taken up by most eukaryotic cell types and sufficiently transferred to the cytoplasm and nucleus. The cellular uptake of ODN can be demonstrated using a fluorochrome-conjugated control ODN [1]. Accordingly, the use of antisense oligonucleotides as therapeutic agents has heralded a new field of genetic pharmacology [2].

During a normal immune response, both Th1 and Th2 cell types are involved in a cross regulatory fashion. It is suggested that an imbalance between these subsets contributes to the development of diseases [3, 4]. Atopic individuals have a particular immune phenotype, producing high levels of IgE and having elevated expression of Th2 lymphocytes that secrete IL-4, IL-5 and IL-13 cytokines [5].

In order to produce IgE, antigen presenting cells, including B-lymphocytes, process allergens and present them to CD4$^+$ T lymphocytes. It has been shown that interaction between CD40 molecule on B cells and CD40L on T cells is critical for T cell dependent isotype switching. Therefore, stimulation of human B cells by anti-CD40 plus IL-4 results in proliferation and IgE production [6, 7]. Since anti-CD40 monoclonal antibody mimics the effect of CD40L, application of anti CD40 mAb plus IL-4 induces IgE production *in vitro* [8, 9]. However, CD40-CD40L contact and an obligatory cytokine signal, either IL-4 or I-13 are necessary components for switching the genetic program of a B cell from IgM (the default antibody) to IgE [10].

Although IL-13 is known as a Th2 cytokine, its production is not restricted to T lymphocytes. Recently, it has been shown that in human B cells, IL-4 and IL-13 mRNA and protein are detected following activation of the cells by platelet activating-factor [11, 12]. Furthermore, during the experiment with purified tonsillar B lymphocytes

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stimulated to produce IgE by adding antibody to CD40 plus recombinant IL-4 in the absence of T cells, there was a significant synthesis of the cytokines IL-6 and IL-13 [13- 15].

Although there are several reports about antisense applications in different diseases such as cancer, there is no direct report on anti IL-13 antisense effects on antibody production [16, 17]. In search of molecules that might induce a therapeutic down regulation of IgE synthesis, which is considered to have a basic effect in atopic diseases, we study the effects of IL-13 antisense ODN on IgE production by tonsillar B-lymphocytes. Since the neutralization of IL-13 may down regulate the IgE synthesis, we would like to determine whether this antisense oligo would have a differential effect on the production of the immunoglobulin isotyes. In the present study, we designed an effective antisense oligo for human IL-13 in order to detect the autocrine effect of IL-13 in cultured B-cells.

MATERIALS AND METHODS

Design and synthesis of antisense. A 20-mer IL-13 antisense ODN was designed to inhibit IL-13 translation according to the entire m-RNA sequence for possible antisense target sites. All the sequences were then compared with each other and with database which contains the complete molecular characteristics of all tested antisense ODN. The sequences that showed relevant cross homologies to other gene bank sequences were excluded. Finally, all remaining candidate sequences were ranked 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 (Canada, Montreal) in phosphothioate form. The preparation was HPLC purified then freeze-dried and kept in -20ºC. According to this sequence, a fluorescence labeled ODN was also synthesized and applied to cell uptake testing.

Preparation of mononuclear cells from tonsils. Fresh human tonsils were aseptically removed and put into Petri dish with 5 ml washing media (RPMI 1640+ 20% FBS) and cut into slices using sterile scalpel. Cells were detached as many as possible by mincing with a syringe part. Isolated cells were collected in washing media into 50 ml tube and let settle to remove tissue clamps. Thirty five ml of cell suspension was underlaid to 15 ml Ficoll-Hypaque (Pharmacia, USA), and centrifuged at room temperature at 700 g for 30 min. Mononuclear cells in interface layer were aspirated into 50 ml tube,

washed 3 times with 50 ml Washing Media for 10 min. Finally, viable nucleated cells were counted using trypan blue staining under light microscopy and adjusted to 5×10^6 cell/ml with washing media.

B-lymphocyte separation. B cells were enriched from mononuclear cells using a magnetic cell sorting kit (MACS, Miltenyi Biotec., USA). In this method, colloidal super paramagnetic micro beads conjugated to mouse monoclonal CD19 antibody were used according to supplier procedure using positive selection columns (MS/RS). Briefly, single cell suspension was washed and resuspended in 80 ml of buffer (PBS pH 7.2, 0.5% bovine serum albumin, 2 mM EDTA) per 10^7 total cells. CD19 micro beads were added (20 µl), mixed well and incubated at 6ºC for 15 min. The column was placed in the magnetic field of an appropriate magnetic cell separator and prepared by washing with a 500-µl buffer. Cell suspension was applied onto the column, negative cells were allowed to pass through, and column was rinsed with buffer (3 × 500 µl). Column was removed from separator and placed on a suitable collection tube. Buffer (5 ml) was pipetted onto the column and flushed out CD19 positive cells using the plunger supplied with the column.

Cell culture for induction of IgE response. Purified B cells were cultured (10^6 cells/ well) in 24 wells cell culture micro plates for 14 days. The culture medium RPMI 1640 was supplemented with L-glutamine (4 mM), penicillin (100 U/ml), streptomycin (100 mg/ml), MEM sodium pyruvate (1 mM), HEPES buffer (15 mM), and 10% heat inactivated fetal calf serum (all from Gibco, UK). Cell cultures were performed at 37ºC in humidified air and 5% CO_2 atmosphere. In order to induce IgE production, cultured cells were stimulated by adding 1 µg/ml of monoclonal antibody to CD40 (Serotec, UK), plus 400 U/ml of recombinant human IL-4 (R and D System, USA).

Antisense cell uptake measurement by flowcytometry. In order to determine the incorporation of IL-13 antisense oligo into the cells, B-lymphocytes were cultured (20,000 cell/well in 24 wells micro plate) in cell culture media in the presence of anti-CD40 monoclonal antibody and rIL-4 (described before). The fluorescent antisense ODN (synthesized by Invitrogen), was added after 2, 4, 8, 18, 24, and 48 hours of culture. Cells were collected at each time course, washed in PBS-BSA 1%, and analyzed using a FACScan Flow cytometer (Becton Dickinson, USA).

Sample collection for antibody assay. Purified B-lymphocytes were cultured in order to induce IgE in three 24-well culture plates up to 14 days. At the first plate (test), IL-13 antisense ODN was added (2 µl) to culture media on days 1 and 3. The second plate was considered as negative control with no oligo, and in the tired one, in order to determine the IL-4 effect; this cytokine was removed from the medium on day 3. For this, the cells were washed and resuspended in fresh media plus antisense oligo and anti CD-40 antibody. All supernatants were collected on days 1, 7, 10 and 14, and stored in aliquots at -20ºC until they were assayed for antibodies.

IgE assay by ELISA. For detection of human IgE an ELISA procedure was already established in the laboratory for detection of human IgE in cell culture supernatants [13]. Briefly, monoclonal rat anti-human IgE (Biosorce, USA, 5 µg/ml in 0.05 M carbonate bicarbonate buffer, pH 9.6) was coated on round-bottom high-binding 96-well plates (Costar, USA) overnight. As for all subsequent washing steps, the plates were washed five times with phosphate-buffered saline containing 0.1% Tween-20. Subsequently, the plates were blocked for 2 hours at 37ºC with 200 µl PBS containing 0.5% gelatin (blocking buffer). After washing, samples were added (100 µl/well) and incubated at room temperature for 2 h or overnight at 4ºC. After washing, 100 µl diluted biotinylated goat anti-human IgE (Biosource, USA) in blocking buffer was added per well and incubated at room temperature for 2 h. Then, plates were washed and incubated for 1 hour with 100 µl/well HRP-streptavidin (Biosource, USA) 1:10,000 dilution in blocking buffer. After washing, the plates were developed with 100 µl/well tetramethyl-benzidine (TMB, Merck, Germany) in 0.1 mol/L sodium acetate, pH 5.5 containing 0.003% H_2O_2. The reactions were terminated by the addition of 50 µl of 2 mol/L H_2SO_4 to each well. Developed color was read at 450 nm in a Bio-Rad 350 plate reader (Bio-Rad, Richmond, Canada). In this experiment, purified human IgE (prepared from Biosorce, USA), diluted in blocking buffer, was used as a standard and standard ranged from 5 to 5,000 pg/ml.

ELISA for IgA, IgG and IgM assay. Immunoglobulin isotype assay was performed by ELISA as follows: Nunc Maxcisorb U-96 well plates were coated with 3 µg/ml goat anti human IgA, IgM or IgG (Serotec, UK) in 0.1 M bicarbonate buffer, pH 8.2. After blocking the wells with blocking buffer (0.1 M Tris, 2 mM $CaCl_2$, 1%

BSA, 0.2% Na azide, pH 8.2), standards (10-300 ng/ml) and samples (1:5 dilution) were applied in separate wells and incubated overnight at 4ºC. At the next stage, specific goat anti-human Ig isotypes conjugated to alkaline phosphatase (Serotec, UK) were added to appropriate plates as the secondary antibody. Enzyme activity was revealed with 2 mM phosphate substrate (Sigma, USA) prepared in 1M TRIS buffer. After 15-30 minutes, developed color was measured at 405 nm by ELISA reader. Standard curves were prepared using serial dilutions of standard human immunoglobulins (Sigma, USA) from 10 to 300 ng/ml. Finally, Ig assay was calculated in ng/ml for each sample.

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

RESULTS

B-cell MACS purification. B cells were separated by MACS CD19 micro beads with about 99% B-cell purity. The purity was evaluated by flow cytometry according to the CD19+ cell staining with fluorochrome conjugated CD19 antibody (Fig. 1).
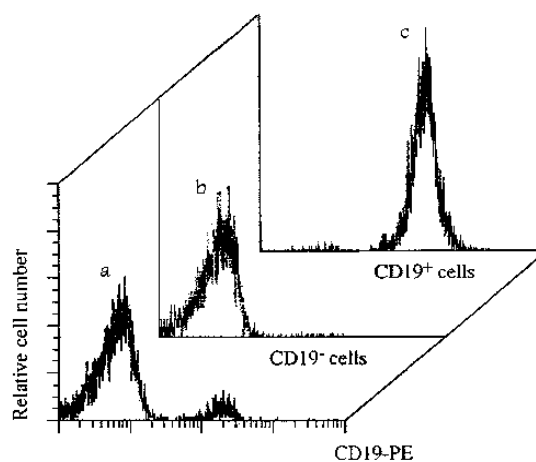
Fig. 1. Purified B-lymphocytes using anti CD19 micro beads. Mononuclear cells are analyzed with flowcytometer according to FL1 positive cells. Three cell preparations are stained with Phycoerythrin conjugated anti-CD19 monoclonal antibody (CD19-PE). a) Ficoll separated mononuclear cells before passing through the column. b) CD19 negative cells passed through the column. c) Magnetically retained CD19 positive cells which are eluted as positively selected cell fraction.
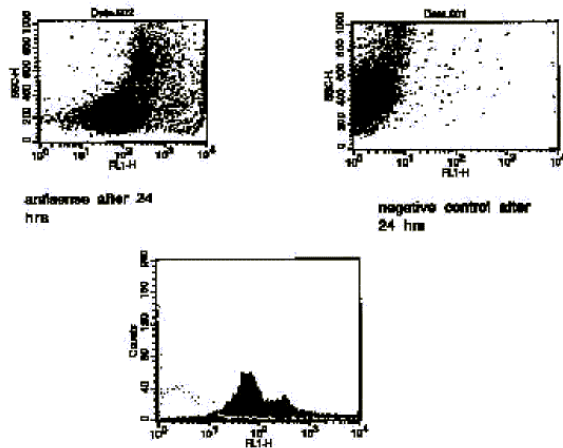
Fig. 2. Purified B-cells are incubated with fluorescence IL-13 antisense oligo for 24 hours and analyzed with flow cytometry (top left). A cell culture plate without antisense oligo is considered as negative control (top right). There is a right shift for positive cells (filled histogram) compared with negative control at the bottom histogram.

Evaluation of fluorescent-oligo cell uptake. Flow cytometric analysis of cultured B cells incubated with fluorochrome conjugated IL-13 antisense in different hours indicated that labeled antisense oligo

can be uptaked by about 90% of cells after 1 h incubation. Evaluation of time course oligo uptake revealed that these fluorescent positive cells are present more than 72 h after incubation. Figure 2 shows 24 h incubation in comparison with negative control.

***Evaluation of secreted immunoglobulins.*** Measurement of different Ig classes in cell culture supernatants collected in test plate, in comparison with negative control, indicated significant decrease in IgE, increase in IgA and no significant changes in IgM and IgG concentrations on different days (Fig. 3). After removal of IL-4 from media on day 3 (plate #3) the IgE levels reduced to zero and IgM and IgG levels decreased significantly (Fig. 4).

DISCUSSION

Cytokines IL-4 and IL-13 are responsible for Th2 cell proliferation. The induction of IgE synthesis in B cells plays a key role in the pathophysiology of diseases such as asthma [17]. Although IL-13 is known as a Th2 cytokine, its production is not
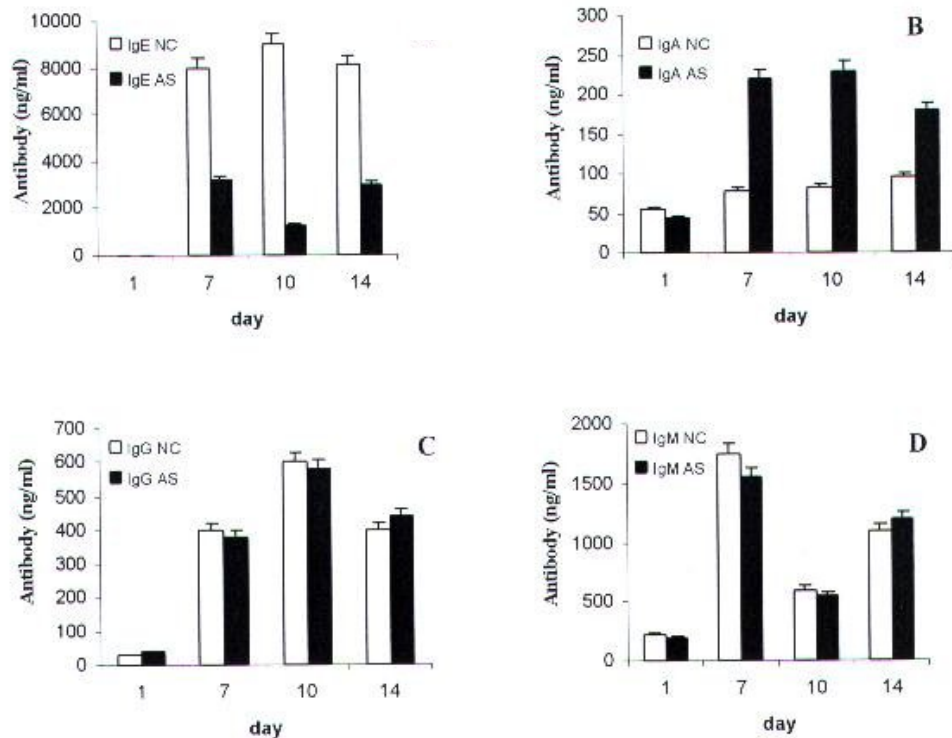
Fig. 3. Ig assay in B cell culture media. Antisense oligo is added twice in test plates (AS), no oligo is added in the control plates (NC). IgE concentrations decrease significantly **(A)**, IgA increases **(B)**, IgG and IgM demonstrate no significant changes on different days **(C and D)**.

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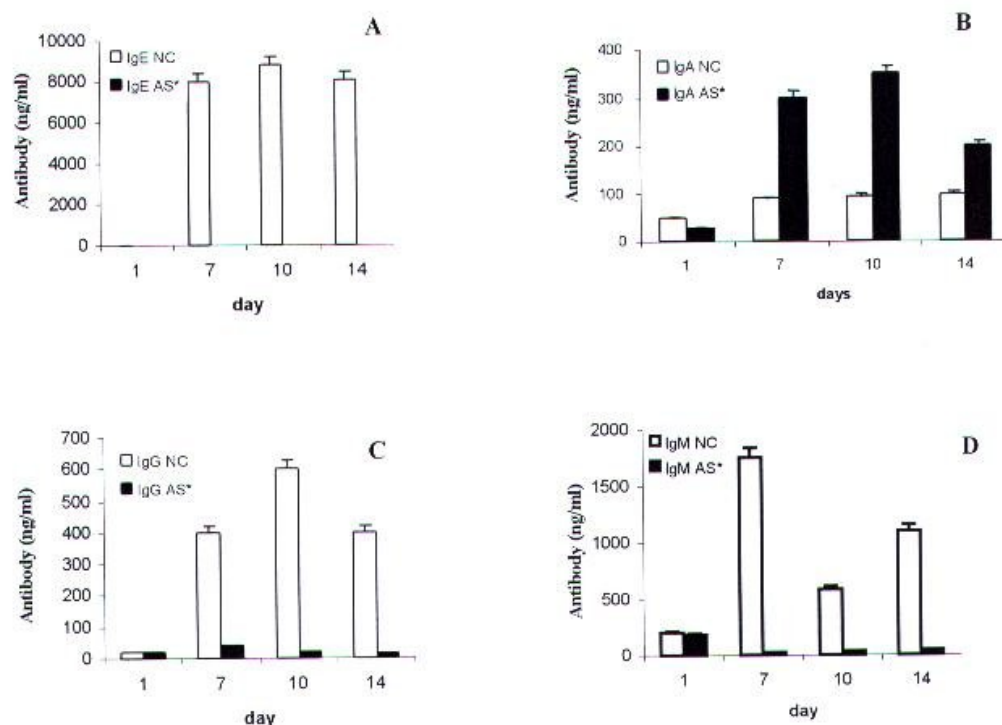
Fig. 4. Ig assay in B cell culture media. Antisense oligo is added and IL-4 is removed in the test plates (AS*). No oligo addition and IL-4 removal were performed in negative control plates (NC). IgE concentrations are zero on different days **(A)**, IgA shows significant increase **(B)**, IgG and IgM demonstrate significant decline **(C and D)**.

restricted to T lymphocytes [11, 14, 18]. It has been suggested that following the ligation of CD-40 and the addition of cytokine, human B-lymphocytes increase the IL-13 m-RNA [18]. Therefore, we cultured purified human tonsillar B-cells in the presence of anti CD-40 monoclonal antibody as a T-B cell interaction substitute plus IL-4 to promote IL-13 secretion. It has been reported that human B-cells produce IL-13 that involves in Ig class switching from IgM to IgE on RNA transcription at unrearranged ε heavy chain locus [5]. We studied IL-13 inhibition and the consequences on immunoglobulin levels. Previous reports indicated the effects of anti IL-13 and anti IL-13 receptor: $\alpha 1$ and $\alpha 2$ monoclonal antibodies in neutralizing IL-13 in B-cell culture and further down regulation effects on IgE production [19-21]. Although these antibodies partially decreased IgE concentration, no investigation was performed on the other Ig classes. Although many reports indicate cytokine specific antisense oligos and their effects on Ig production, there are no reports about the role of human IL-13 antisense on Ig levels [22]. Accordingly, we applied an antisense ODN, which was already designed for human IL-13 inhibition in our laboratory.

The preliminary outcomes of our investigation on human IL-13 antisense oligo demonstrated that this oligo could be entered efficiently into the cell endosomes, released in the cytoplasm after 1 hour and remained for at least 72 h. We also developed an appropriate cell culture condition and defined the correct time course for oligo applications. We demonstrated that twice addition of oligo on days 1 and day 3 could inhibit the IL-13 production from B cells.

However, IL-4 and IL-13 alone are sufficient to drive ε germ line transcription in cultured B cells [23, 24]. The IL-13 blocking by antisense oligo resulted up to 85% decrease in IgE level that suggests its superiority. Conversely, the IgM and IgG levels appeared to be independent of IL-13, and there is a significant decrease in concentration after IL-4 removal.

Although the effectiveness of targeting IL-13 in Ig class switching is not well described [25, 26], our preliminary results demonstrated that the inhibition of this cytokine by antisense oligo could be useful to block IgE and augment IgA. Since the inhibition of ε chin translation due to IL-13 inhibition leads to isotype switching to IgA [27, 28], we conclude that

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antisense oligo application can be a useful therapeutic tool in IgE mediated diseases.

In contrast to IgE and IgA classes, we found that no significant changes were made in IgM and IgG levels using IL-13 antisense oligo, suggesting no obvious effect of IL-13 on IgM and IgG production. We also demonstrated that IL-4 removal in cell culture media leads to significant decline in IgM and IgG concentrations suggesting potential effects of IL-4 on IgM and IgG secretion. Since the complete blocking of IgE appears to be dependent on both IL-13 and IL-4, further studies are needed to define precise IL-13 and IL-4 mechanisms involved in Ig isotype switching and its potential therapeutic application in asthmatic patients. Also, IL-4 function on IgG and IgM secretion in the absence of IL-13 needs to be investigated.

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