

SPECIFIC INHIBITION OF LYMPHOKINE BIOSYNTHESIS
AND AUTOCRINE GROWTH USING ANTISENSE
OLIGONUCLEOTIDES IN Th1 AND Th2
HELPER T CELL CLONES

By ANNICK HAREL-BELLAN,* SCOTT DURUM,* KATHRYN MUEGGE,*
ABUL K. ABBAS,[†] AND WILLIAM L. FARRAR*

*From the *Laboratory of Molecular Immunology, Biological Response Modifier Program, National Cancer Institute, Frederick Cancer Research Facility, Frederick, Maryland 21701; and the*

[†]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

The helper/inducer class of T lymphocytes has been recently divided into two nonoverlapping subsets on the basis of their lymphokine secretion and requirement for growth (1-3). Helper clones from the Th1 subset produce IL-2 upon antigen or lectin stimulation, whereas clones from the Th2 subset produce IL-4, also known as B cell stimulating factor 1 (BSF-1).¹ Both subsets respond to IL-2, and both express, with minor quantitative differences, receptors for both lymphokines (4). But antibodies directed against IL-2 inhibit only the antigen-stimulated growth of Th1 cells, whereas antibodies directed against IL-4/BSF-1 inhibit only the growth of Th2 cells (4). To confirm this classification, we have used a novel approach, which allows the specific inhibition of the biosynthesis of each respective lymphokine. Antisense inhibition of specific protein synthesis is achieved by saturating the cell with a nucleotide whose sequence is complementary to a portion of the mRNA encoding the protein (5-10). The intracellular annealing of the complementary sequence to the message prevents the synthesis of the protein. The complementary sequence can be introduced into the cell by transfection of a plasmid in which the gene encoding the protein is in opposite orientation with respect to the promoter (6-10). In that case, an intracellular excess of antisense RNA is obtained, and an intracellular RNA/RNA complex is formed. Alternatively, chemically synthesized short complementary DNA sequence can be used (11-19). These synthetic oligonucleotides actively penetrate into the cells, achieving relatively high intracellular levels (19). The advantage of using such a strategy for lymphocytes is that, besides the fact that the low efficiency transfection step can be avoided, the resultant intracellular duplex is a DNA/RNA hybrid. Such duplexes are substrates for an enzymatic activity, RNase H, which

A. Harel-Bellan is a Chargee de Recherches (Centre National de la Recherche Scientifique, France) supported by a fellowship from the Fogarty Center. Address correspondence to A. Harel-Bellan, Institut Gustave Roussy, Laboratoire d'Immunologie, Bariment de Recherches, 39 rue Camille Desmoulins, 94800 Villejuif, France.

¹ Abbreviation used in this paper: BSF, B cell stimulating factor.

specifically degrades the RNA strand in a DNA/RNA duplex (20–22). Therefore, the efficiency of the inhibition is potentially enhanced.

In this study, we have used two synthetic oligonucleotides, complementary to the 5' end of the mRNA coding for mouse IL-2 and mouse IL-4 (BSF-1). These sequences correspond to portions of the leader sequence of the molecules. Proliferation of cells from a Th1 clone (D1.1) was inhibited by the IL2 antisense oligonucleotide, whereas cells from a Th2 helper clone, D10 (23), were only inhibited by the IL-4 antisense oligonucleotide. The inhibition was reversed by addition of exogenous lymphokine in both cases. The inhibition was accompanied by a decrease in the steady-state level of the corresponding lymphokine mRNA, suggesting a possible degradation of the message through an RNase H like activity.

Materials and Methods

Cell Lines. The D1.1 cell line (2) is a helper T cell from the Th1 subset. It was maintained in 10% FCS RPMI supplemented with 1% glutamine and antibiotics (Gibco Laboratories, Grand Island, NY) and 1% nonessential amino acids (Gibco Laboratories), and stimulated weekly with 100 µg/ml of rabbit IgG presented by irradiated (1,200 rad) BALB/c spleen cells depleted of red cells by a short hypotonic shock. The D10.G4.1 (D10) (23) cell line is a helper T cell from the Th2 subset. It was maintained in RPMI 10% FCS supplemented with 1% glutamine and antibiotics and stimulated weekly with Conalbumin (100 µg/ml) and mitomycin C-treated C3H/HeJ spleen cells.

Oligonucleotides. Oligonucleotides IL-2 antisense (5'-CTGCATGCTGTACAT-3'), IL-4 antisense (5'-GGGGTTGAGACCCAT-3'), and IL-4 sense (5'-ATGGGTCTCAACCCC-3') were synthesized on a multiple column DNA synthesizer (model 8700; Biosearch, San Rafael, CA) purified on acrylamide/urea gels followed by electro-elution and several cycles of ethanol precipitation. Oligonucleotides were finally resuspended in PBS before use.

Proliferation Assays. Cells were seeded in microtiter plates in RPMI supplemented as indicated above and with 1% FCS (65°C heat inactivated) and in the presence or absence of various doses of oligonucleotides. After 2 h of incubation at 37°C, the stimulant was added (100 µg/ml of rabbit IgG plus irradiated APCs for D1.1; 100 µg/ml Conalbumin plus mitomycin C-treated APCs for D10) or not for the control, and the concentration of serum was raised to 5%. After a period of 3 d, cultures were washed with RPMI in order to avoid any nonspecific inhibition due to the possible presence of free thymidine from degraded oligonucleotide (three times in 250 µl followed by centrifugation for 3 min at 1,200 rpm); 1 µCi of [³H]thymidine (6.7 Ci/mmol; New England Nuclear, Boston, MA) in 10% FCS RPMI was added in each well and cultures were incubated overnight at 37°C. Cultures were harvested on a Skatron AS (Heggtopen, Norway) cell harvester and incorporated radioactivity was quantified by scintillation counting. All assays were carried out in triplicate. For reversal experiments, 10 U/ml of human IL-2 (Cetus Corp., Emeryville, CA), or 200 U/ml of mouse IL-4 (Immunex Corp., Seattle, WA), or up to 50% of crude D10 conditioned supernatant was added after the preincubation with oligonucleotides.

Northern Analysis. D1.1 or D10.G2 cells (15×10^6 in 5 ml of 1% 65°C heat-inactivated FCS/RPMI) were preincubated in the presence or absence of 5 µM antisense IL-2 or IL-4 oligonucleotide, respectively, for 2 h as described above. 10 µg/ml of Con A (D1.1) or 10 µg/ml of Con A plus 1 U/ml of mouse rIL-1 (kind gift of Dr. W. Benjamin, Hoffmann-La Roche, Inc., Nutley, NJ) (D10) was added and the serum concentration was raised to 5%. After an overnight period of incubation, cells were harvested, washed once in PBS, and resuspended in guanidium-isothiocyanate followed by purification of RNA on a standard cesium chloride gradient.

Equal amounts of RNA (15 µg of total RNA) were denatured with 6% formaldehyde for 10 min at 55°C in 1× MOPS (20 mM morpholine propane sulfonic acid, pH 7.1, 5 mM sodium acetate, 1 mM EDTA) and 50% deionized formamide (Fluka Chemical Co., Hauppauge, NY), quickly chilled on ice, and size fractionated on 1% agarose gels containing 6%

formaldehyde. After soaking of gels in $20\times$ SSC ($1\times$ SSC = 150 mM sodium chloride, 15 mM trisodium sodium citrate, pH 7), RNA was transferred onto nitrocellulose filters (BA 85; Schleicher & Schuell, Inc., Keene, NH) by capillary blotting in $20\times$ SSC using standard procedures (24). Filters were then baked 2 h under vacuo at 80°C .

All probes used in this study were purified inserts, isolated after appropriate restriction on 1% low-melting-point agarose (Bethesda Research Laboratories, Bethesda, MD), ^{32}P -labeled to $2\text{--}5\times 10^8$ cpm/ μg by primer extension using the Polymeraid labeling kit (PLS) and [^{32}P]dCTP (3,000 Ci/mM; Amersham Corp., Arlington Heights, IL), and was purified on G50 columns (Boehringer Mannheim Biochemicals, Indianapolis, IN). The mouse IL-2 probe was a gift from Frank Lee (DNAX Inc., Palo Alto, CA). The mouse IL-4 probe was a gift from K. Arai (DNAX, Palo Alto, CA). The mouse actin probe was a gift from Dr. K. O'Connell, PRI, Frederick, MD; the ribosomal RNA probe was a gift from Dr. D. Radzioch, PRI. Filters were soaked for 2–24 h in prehybridization buffer ($5\times$ SSC, 50% formamide, $1\times$ Denhardt's solution, 20 mM Tris, pH 7.4, 0.2 mg/ml salmon sperm DNA, 5% dextran sulfate), and hybridized with 5–10 ng/ml of labeled probes in the same medium for 20–48 h at 42°C . Filters were washed several times in $2\times$ SSC, 0.1% SDS at room temperature, one to three times 15 min in $0.1\times$ SSC, 0.1% SDS at 50°C , and exposed to Kodak XAR-5 films with Cronex lighting plus intensifying screens (Dupont Co., Wilmington, DE) at -70°C for 3–48 h. For relative quantities of hybridized radioactive probe, autoradiographs were scanned using a densitometer and the relative intensities of the bands were estimated by weighing the densitometer profiles. Hybridized radioactive probe was then removed by immersion of the nitrocellulose filters in boiling water. Filters were checked before use with other probes.

Results

Specific Inhibition of the Proliferation of Th1 or Th2 Cell Lines by IL-2 or IL-4 Antisense Oligonucleotides. Previous studies by our laboratory and others (16–19) have shown that short oligonucleotides readily penetrate lymphoid or myeloid cells, reaching significant levels within a few hours. Therefore, cells from the D1.1 clone, a murine Th1 helper clone (1) or from the D10 clone, a murine Th2 helper clone (23), were preincubated for 2 h with various doses of anti-IL-2 or anti-IL-4 antisense oligonucleotides, and activated with antigen and irradiated APCs. Proliferation was measured at day 3, using a thymidine incorporation assay as described in the Materials and Methods. D1.1 cell proliferation was inhibited by the anti-IL-2 antisense oligonucleotide, whereas D10 cells were inhibited by the anti-IL-4 oligonucleotide (Fig. 1). In both systems, a maximal inhibitory effect (90–100%) was reached between 5 and 10 μM of oligonucleotide. In both systems, the nonhomologous oligonucleotide against the reciprocal interleukin did not have such an antiproliferative effect, indicating that the inhibition was specific and not due to a toxicity of the oligonucleotides.

The Inhibition Can Be Specifically Reversed by the Addition of Exogenous Lymphokine. The specificity of the inhibition was further assessed by reversal experiments. We reasoned that if the inhibition was due to the lack of lymphokine biosynthesis, the inhibition should be reversed by the addition of exogenous IL-2 or IL-4. Therefore, antisense-treated D1.1 or D10 cells were cultured in the presence or absence of exogenously added IL-2 or IL-4, respectively. The results shown in Fig. 2 indicated that exogenous rIL2 was able to significantly reverse the proliferative blockade of antisense IL-2-treated D1.1 cells, further demonstrating that the inhibition was specific and not due to a toxic effect of the oligonucleotide. Similarly, exogenously added rIL-4 was able to overcome the inhibition of D10 proliferation by antisense IL-4

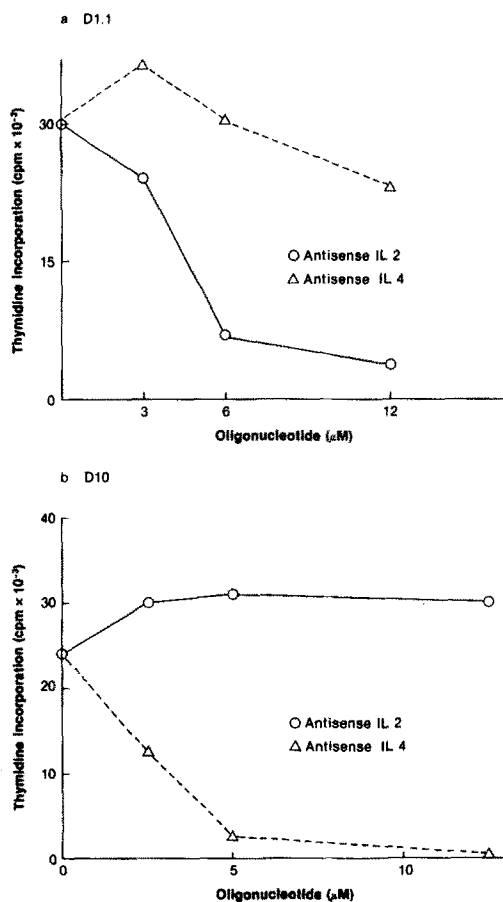


FIGURE 1. Inhibition of Th clones proliferation by antisense oligonucleotides. D1.1 (a) or D10 (b) cells were incubated 2 h with indicated doses of IL-2 or IL-4 antisense oligonucleotides, activated with antigen plus APCs, and thymidine pulsed 3 d later. The figure shows the result of a typical experiment, and three to five independent experiments gave the same results.

oligomer. However, in the case of the D10 cells, this reversal effect was observed only in the low range of oligonucleotide concentration. A partial reconstitution was also observed when a crude supernatant from antigen-activated D10 cells was used instead of rIL4 (data not shown). This suggests that this lack of complete reconstitution was not related to an intracellular induction by IL-4 of other indispensable growth factors that could be present in the crude supernatant. A sense control oligonucleotide did not have any effect on D10 cell proliferation in the range of doses used for the antisense (data not shown). It is likely that, at high doses, the antisense IL-4 oligonucleotide caused some nonspecific inhibition. This is confirmed by the slight nonspecific inhibition observed on D11 cells using the antisense IL-4 oligonucleotide, and could be related to the sequence itself.

Treatment with the Antisense Oligonucleotides Results in Reduced Steady-State Level of Lymphokine Message. To gain insight into the mechanism by which the antisense oligonucleotides inhibited the autocrine growth of D1.1 and D10 cells, we performed Northern analysis of the steady-state levels of lymphokine messages in these cells. D1.1 or D10 cells were incubated with antisense IL-2 or IL-4 oligonucleotides, respectively, and activated with Con A (D1.1) or Con A plus IL-1 (D10). After an overnight

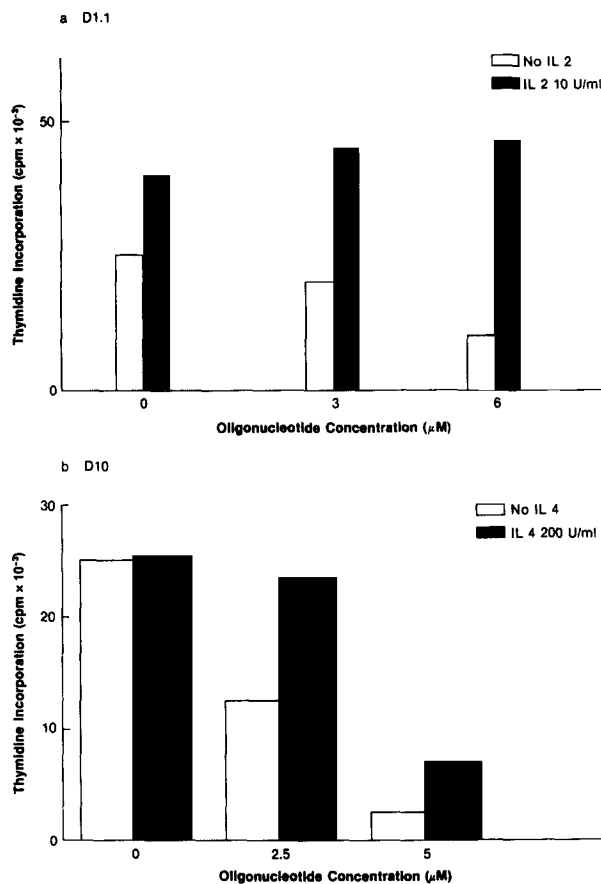


FIGURE 2. Reversal of inhibition by exogenous lymphokines. D1.1 (a) or D10 (b) cells were incubated with or without IL-2 or IL-4 antisense oligonucleotides as indicated, and activated with antigen and APCs in the presence or absence, as indicated, of exogenous lymphokine. Cultures were thymidine pulsed 3 d later. The figure shows the result of a typical experiment, and two to three independent experiments gave identical results.

period of culture, cells were lysed and total RNAs were extracted and analyzed using IL-2 and IL-4 cDNA probes (Fig. 3). The antisense IL-2-treated D1.1 cells did not express detectable level of IL-2 message, when compared with untreated controls similarly activated with Con A plus IL-1. Conversely, the antisense IL-4-treated D10 cells showed a significantly decreased level of IL-4 message (fivefold decrease). No IL-4 message was detected in any of the D1.1 RNA samples and no IL-2 message was detected in any of the D10 RNA samples (data not shown). The decrease in lymphokine message was specific, since the steady-state level of actin message was not impaired by the treatment with oligonucleotides (Fig. 3). Furthermore, the level of rRNA was also unchanged in these samples (data not shown).

Discussion

In prokaryotic cells, specific blockade of the expression of a gene has been one of the major tools to assess the function of its product, through the use of deletion or thermosensitive mutants. In mammalian cells, the difficulty of deriving mutant cell line has prompted the search for other means of specific gene product deletion. A possible solution was derived from the study of gene regulation in bacteria, where

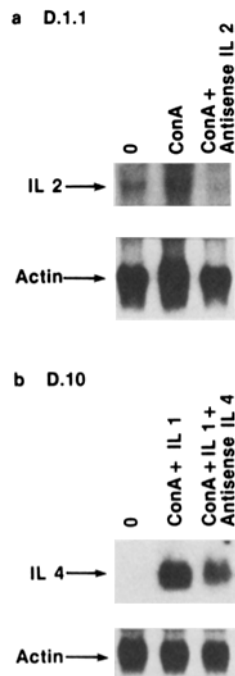


FIGURE 3. The steady-state level of lymphokine mRNA is decreased in the presence of antisense oligonucleotides. D1.1 (a) or D10 (b) cells were incubated with or without IL-2 or IL-4 antisense oligonucleotides as indicated, and activated with Con A or Con A plus IL-1 as indicated. After an overnight period of culture, cells were washed and RNAs were extracted and purified. RNAs were electrophoresed on denaturing agarose gels, blotted, and analyzed for the level of IL-2, IL-4, or actin mRNA, as indicated.

the expression of certain genes is regulated by naturally occurring complementary RNAs: such antisense RNAs are thought to hybridize with the normal message and therefore to block its recognition and processing by the translational apparatus of the cell (reviewed in reference 5).

Recent studies have shown that artificial introduction of antisense plasmids into eukaryotic cells resulted in a specific inhibition of the expression of exogenous as well as endogenous cellular genes (6-10). The caveats of this technique are that: (a) One needs to be able to efficiently introduce exogenous DNA in the cells; (b) There is a general requirement to achieve a 100-fold excess of the antisense over the endogenous sense message; and (c) In the specific case of genes related to cell proliferation, an inducible blockade is required. Inducible promoters such as the dexamethasone-inducible MMTV promoter, have been successfully used in mouse fibroblasts for specific inhibition of the *c-fos* gene (10). This promoter, however, cannot be universally used, since dexamethasone is, by itself, inhibitory for growth of most lymphoid cells. Alternatively, synthetic oligonucleotides complementary to viral genes have been shown to prevent infection of cells by several viruses, without affecting normal cell growth (11-15). In addition, we and others (16-19) have specifically deleted the expression of *c-myc* gene in T lymphocytes or in myeloid cells. In the present study, we demonstrate the successful use of this strategy for the selective deletion of secretory proteins in lymphocytes.

Studies of the lymphokine production and requirement for growth of cloned helper T cell have shown the existence of two subsets, the Th1 and the Th2 subset. Using antibodies directed against these two lymphokines, it has been demonstrated that

the Th1 type produces IL-2 and proliferates using IL-2 as an autocrine factor, whereas the Th2 type helper clones produces IL-4 and not IL-2, and proliferates using IL-4 as an autocrine factor. The two subsets are not overlapping, the Th1 type helper clones being inhibited only by anti-IL-2 antibodies, whereas the Th2 type helper clones are inhibited only by anti-IL-4 antibodies. We here confirm these observations with the antisense strategy, using complementary, or antisense, short oligodeoxynucleotides. We show that antisense directed against the IL-2 message was able to inhibit only a Th1 helper clone proliferation, whereas antisense directed against the IL-4 message inhibited only cells from a Th2 clone. The inhibition was not due to a toxic effect of the oligonucleotides, since no inhibition was observed with the antisense IL-4 on IL-2-dependent T cells, and vice-versa.

Furthermore, the inhibition could be reversed by exogenous addition of the lymphokine, although in the case of the antisense IL-4 oligonucleotide at high doses, the reversal was only partial. This could suggest an intracellular effect of the lymphokine that could not be reproduced by the addition of extracellular IL-4. It is likely, however, that this partial reversal reflects a nonspecific inhibition by high doses of antisense IL-4, which is not detected with the antisense IL-2, but has been observed in various systems.

The antisense oligonucleotides were originally thought to act through a specific inhibition of mRNA translation, on the basis of the results of several *in vitro* studies (25-28). However, it has recently been suggested that in certain systems, the protein synthesis inhibition could in fact result from the degradation of the relevant mRNA by an enzymatic activity known as RNase H (22). RNase H specifically degrades the RNA strand in a DNA/RNA duplex. In this study, we show that the inhibition of cell proliferation was accompanied by a lower steady-state level of the relevant message in each cell line. It is not known, at this point, if this lower level of message results from a transcriptional or post-transcriptional event. It could be due to a feedback effect of the disappearance of the protein, as it has been shown with mutated globin genes, for example (29). However, a likely hypothesis is that the lymphokine messages are degraded by an RNase H-like activity.

This type of study, involving the actual and specific inhibition of a lymphokine, could prove helpful in understanding the respective role of each of the helper T cell subsets in the different immunological situations in which helper T cells are known to be involved. Antibody-mediated inhibition of lymphokines often does not give consistent results. Antibody inhibition relies on the fact that the antibody can inhibit the binding of the lymphokine to the cellular receptor. The inhibition therefore depends on the relative affinity of the antibody versus the physiological ligand for the receptor. Furthermore, no effect would be obtained on the potential intracellular action of the lymphokine in the producing cell. The antisense oligonucleotide strategy, which allows the inhibition of the actual biosynthesis of the lymphokine, could overcome these difficulties. In addition, antisensing lymphokines could have potential therapeutic applications in the case of autocrine cancer cells, as it is already envisioned in the case of viral disease (11-15).

Summary

T helper cells have recently been divided into two subsets. The Th1 subset secretes and responds to IL-2 in an autocrine manner. The Th2 subset upon mitogen

or antigen stimulation releases IL-4. Here we describe a novel technology that allowed us to confirm this distinction. We have used synthetic oligonucleotides complementary to the 5' end of mouse IL-2 and IL-4 to specifically block the biosynthesis of IL-2 or IL-4 in two murine helper T cell clones from the Th1 or Th2 subset. We show that the antisense IL-2 oligonucleotide inhibited the proliferation of the Th1 clone and had no effect on the Th2 clone. In parallel experiments, the antisense IL-4 oligonucleotide blocked the proliferation of the Th2 clone and not the proliferation of the Th1 clone. The inhibition was significantly reversed in both cases by the addition of the relevant lymphokine (IL-2 in the case of the Th1 clone, IL-4 in the case of the Th2 clone). Northern analysis, using cDNA probes specific for the two lymphokines, showed a decrease in the steady-state level of the relevant lymphokine mRNA, suggesting the specific degradation of the mRNA by an RNase H-like enzymatic activity.

This strategy, which allows the specific blockade of the biosynthesis of a lymphokine, could be useful for future studies on the role of each T helper subset in physiological immune responses.

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