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# CD4 Expression Decrease by Antisense Oligonucleotides: Inhibition of Rat T CD4$^+$ Cell Reactivity

MANEL RABANAL,[1] ÀNGELS FRANCH,[1] VÉRONIQUE NOÉ,[2] CARME PELEGRÍ,[1] CARLOS J. CIUDAD,[2] CRISTINA CASTELLOTE,[1] and MARGARIDA CASTELL[1]

## ABSTRACT

**In previous studies, we have demonstrated the inhibition of CD4 expression in rat lymphocytes treated with phorbol myristate acetate (PMA) by antisense oligonucleotides (AS-ODNs) directed against the AUG start region of the *cd4* gene. The aim of the present study was to inhibit CD4 expression in lymphocytes without promoting CD4 synthesis and to determine the effect of this inhibition on CD4$^+$ T cell function. Four 21-mer ODNs against the rat *cd4* gene (AS-CD4-1 to AS-CD4-4) were used. Surface CD4 expression was measured by immunofluorescence staining and flow cytometry, and mRNA CD4 expression was measured by RT-PCR. T CD4$^+$ cell function was determined by specific and unspecific proliferative response of rat-primed lymphocytes. After 24 hours of incubation, AS-CD4-2 and AS-CD4-4 reduced lymphocyte surface CD4 expression by 40%. This effect remained for 72 hours and was not observed on other surface molecules, such as CD3, CD5, or CD8. CD4 mRNA expression was reduced up to 40% at 24 hours with AS-CD4-2 and AS-CD4-4. After 48 hours treatment, CD4 mRNA decreased up to 27% and 29% for AS-CD4-2 and AS-CD4-4, respectively. AS-CD4-2 and AS-CD4-4 inhibited T CD4$^+$ cell proliferative response upon antigen-specific and unspecific stimuli. Therefore, AS-ODNs against CD4 molecules inhibited surface and mRNA CD4 expression, under physiologic turnover and, consequently, modulate T CD4$^+$ cell reactivity.**

## INTRODUCTION

CD4 IS A 55–59 kDa nonpolymorphic membrane glycoprotein expressed on T helper (Th) lymphocytes that stabilizes the physical interactions between CD4$^+$ T cells and antigen-presenting cells (APC) by binding to the MHC II expressed on APCs (Janeway, 1989; Cammarota et al., 1992). Strategies binding CD4 avoid the interaction between Th and APC and, therefore, inhibit the immune response. In this regard, anti-CD4 monoclonal antibodies (mAb) efficiently induce immune tolerance in the treatment of human and experimental autoimmune diseases (Mauri et al., 1997; Isaacs et al., 1999; reviewed in Nepom, 2002) and in the prevention of transplantation rejection (Mottram et al., 1998; Motoyama et al., 2000; reviewed in Adler and Turka, 2002). Anti-CD4 mAb therapy prevents and improves rat adjuvant arthritis (Pelegrí et al., 1995, 1996) associated with a decrease in Th1-like cells (Pelegrí et al., 2001). In addition, it has proved efficient in other animal models of rheumatoid arthritis (Van den Broek et al., 1992; Chu and Londei, 1996; Mauri et al., 1997) and in clinical studies (Van der Lubbe et al., 1997; Schulze-Koops et al., 1998). In rheumatoid arthritis, preliminary results of open-label trials with two humanized nondepleting anti-CD4 mAb suggest a clinical benefit with a transient reduction in CD4$^+$ T cells (Keystone, 2002). However, these treatments may induce an anti-mAb response that neutralizes the drug and so disables the treatment after several doses (Pelegrí et al., 1996).

[1]Department of Physiology–Division IV, and [2]Department of Biochemistry and Molecular Biology–Division IV, Faculty of Pharmacy, University of Barcelona, Spain.

Gene therapy appears to hold promise for more specific treatments. Gene therapy in autoimmune diseases may replace or compensate for defective or missing genes, deliver immunomodulating molecules, and interfere with signaling processes involved in autoimmune reactions (reviewed in Robbins et al., 2003). Immune responses are initiated by the presentation of antigen T lymphocytes and require the interaction between costimulatory molecules on the APC and T lymphocyte. Interfering costimulation not only inhibits the immune response but also induces anergy in the epitopes presented in the absence of costimulation (reviewed in Robbins et al., 2003). Antigen-specific anergy would have many advantages for the treatment of autoimmune disorders. Antisense oligonucleotides (AS-ODNs) may be a powerful tool to selectively inhibit the expression of a target protein involved in autoimmune disorders.

In previous studies, we have demonstrated that AS-ODNs reduce CD4 expression in rat lymphocytes in which CD4 synthesis is stimulated by phorbol myristate acetate (PMA) (Rabanal et al., 2002). The aim of the present study was to inhibit CD4 expression in rat T lymphocytes by AS-ODNs without promoting CD4 synthesis and to assess the effect of this inhibition on T $CD4^+$ cell proliferative response.

## MATERIALS AND METHODS

### *AS-ODNs*

Four 21-mer oligonucleotides (AS-CD4-1 to 4) against the initiation of translation of the *cd4* rat mRNA (Clark et al., 1987) were designed. The sequences of these AS-ODNs are shown in Table 1. AS-CD4-1 was totally phosphothioate (PS) modified to increase its resistance to nuclease degradation. AS-CD4-2, AS-CD4-3, and AS-CD4-4 were PS-modified at the 3′-end and 5′-end and the internal pyrimidine residues (partially PS-modified) (Peyman and Uhlmann, 1996). Three control ODNs were designed: ODN-C1 was totally PS-modified, and its sequence corresponded to a scrambled AS-CD4-1; ODN-C2, a partially PS-modified ODN, was a mismatch (four bases) of AS-CD4-2; ODN-C4, a partially PS-modified ODN, was a mismatch (four bases) of AS-CD4-4. All these ODNs were synthesized by TIB Molbiol Syntheselabor (Berlin, Germany).

### *Animals*

Female Wistar rats (Charles River, Barcelona, Spain) weighing 180–200 g were used as the lymphocyte source. The study was in compliance with the guidelines for the care and use of laboratory animals approved by the Ethics Commission for Animal Experimentation of the University of Barcelona.

### *Lymphocyte isolation and culture*

Samples were obtained early in the morning (resting phase of rats) when the circadian rhythm of CD4 surface expression was minimal, and the amount of blood Th lymphocytes was almost maximal (Pelegrí et al., 2003). To study CD4 mRNA expression, peripheral blood lymphocytes (PBL) isolated by Nycoprep® gradient centrifugation (Nycomed, Oslo, Norway) were used. To study CD4 surface expression, spleen lymphocytes were isolated as described elsewhere (Rabanal et al., 2002). Cells ($2 \times 10^5$/200 $\mu$l) were cultured in RPMI 1640 medium supplemented with 1% fetal bovine serum (FBS), 50 $\mu$M 2-mercaptoethanol, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, 2 mM L-glutamine, 1% nonessential amino

TABLE 1. SEQUENCES OF ANTISENSE OLIGONUCLEOTIDES AND CONTROL OLIGONUCLEOTIDES USED IN THIS STUDY, INCLUDING POSITION OF TARGET SITES

| *Name* | *Sequence (5′ → 3′)* | *Target sequence* |
|---|---|---|
| AS-CD4-1 | G*A*A*A*G*A*G*A*A*G*C*C*T*C*G*G*C*A*C*A*T* | AUG start codon (+1 → +21) |
| AS-CD4-2 | G*A*A*A G A G A A G C*C*T*C*G G C*A*C*A*T* | AUG start codon (+1 → +21) |
| AS-CD4-3 | C*A*T*G G T*G G C*C*T*G C*T*T*G A G C*G*G* | AUG start codon (−18 → +3) |
| AS-CD4-4 | C*G*G*C*A C*A T*G G T*G G C*C*T*G C*T*T*G* | AUG start codon (−13 → +8) |
| ODN-C1 | A*G*G*C*T*A*A*C*G*A*T*C*C*A*C*A*G*A*G*G*A* | None |
| ODN-C2 | G*A*A*AGAGAAGC*C*T*GC*C*GA*C*A*T* | None |
| ODN-C4 | C*G*G**AC*AT*GGT*C*C*GGT*GC*T*T*G* | None |

*PS modifications.

acids, and 1% sodium pyruvate (Bio-Whittaker Europe, Verviers, Belgium) (RPMI complete medium) for 1–3 days.

### *ODN treatment*

ODNs were mixed with the cationic liposome DOTAP (Roche Diagnostics, Barcelona, Spain) for 20 minutes at ambient temperature and then added to lymphocyte cultures in 20 $\mu$l TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). As controls, lymphocytes were treated either with DOTAP alone or with the same volume of TE buffer (untreated cells).

### *Immunofluorescence staining and flow cytometry*

To quantify CD4 surface expression, FITC-conjugated W3/25 mAb (mouse IgG1, antirat CD4 mAb) was used. To determine cell surface CD8, CD5, and CD3 expression, the following mAbs were used: FITC-conjugated OX8 mAb (mouse IgG1, antirat CD8) (Labgen, Barcelona, Spain), FITC-conjugated OX19 mAb (mouse IgG1, antirat CD5) (Labgen), and 1F4 mAb (mouse IgG1 antirat CD3) (PharMingen, Hamburg, Germany), respectively. Goat antimouse FITC immunoglobulin (Sigma, Barcelona, Spain) was used as secondary antibody in the last mAb.

Antibodies were added to the cells at previously established saturating concentrations and incubated for 30 minutes at 4°C with phosphate-buffered saline (PBS) with 0.1% sodium azide and 2% FBS. After washing, lymphocytes were fixed in 0.5% p-formaldehyde in NaCl solution (0.9%) and stored at 4°C in the dark until analyses were performed. Viable cells with the forward and side-scatter characteristics of lymphocytes were analyzed on an Epics XL Flow Cytometer (Coulter Corp., Hialeah, FL). Cytometer software (System II Software v. 3.0, Coulter Corp.) allowed calculation of the mean fluorescence intensity (MFI), which was proportional to the amount of specific target on cell surface (Arosa and de Sousa, 1995; Jason and Inge, 2001). Results are expressed as the percentage of inhibition according to the following formula:

$$\frac{\text{MFI of untreated lymphocytes} - \text{MFI of ODN lymphocytes}}{\text{MFI of untreated lymphocytes}} \times 100$$

### *Toxicity studies*

Cell viability in the presence of ODNs and DOTAP was determined by flow cytometry using propidium iodide (PI) and fluorescein diacetate (FDA) (Sigma). Living cells were positive for FDA and negative for PI (Darzynkiewicz et al., 1994).

### *Quantitative RT-PCR*

Total RNA was extracted both from ODN-treated and untreated PBLs using the Ultraspec™ RNA reagent (Biotecx, Barcelona, Spain) in accordance with the manufacturer's instructions. cDNA was synthesized in a 20-$\mu$l reaction mixture containing 1 $\mu$g RNA, 125 ng random hexamers (Boehringer Mannheim, Barcelona, Spain), 10 mM dithiothreitol, 20 U RNAsin (Promega, Barcelona, Spain), 0.5 mM dNTPs (Applichem, Madrid, Spain), 4 $\mu$l of 5× RT buffer, and 200 U MLV RT (last two from BRL, Barcelona, Spain). The reaction mixture was incubated at 37°C for 60 minutes, and 5 $\mu$l of the cDNA mixture was used for PCR amplification.

PCR reactions were carried out as follows. A standard 50-$\mu$l mixture contained 5 $\mu$l of the cDNA mixture, 4 $\mu$l of 10× PCR buffer ($Mg^{2+}$ free), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 $\mu$Ci of $\alpha^{32}$P-dATP (3000 Ci/mmol) (Amersham, Madrid, Spain), 2.5 U Taq polymerase (GIBCO, Barcelona, Spain), and 500 ng of each primer. The CD4 primers used were 5′-TCCCAGCGTCGTGATTAGTG-3′ and 5′-CACTGTGTGGGCTACATTC-3′ and, for hypoxanthine phosphoribosyl transferase (HPRT) mRNA, which was used as control, 5′-TCCCAGCGTCGTGATTAGTG-3′ and 5′-GGCTTTTCCACTTTCGCTGA-3′.

The reaction mixture was separated in two phases by a solid parafilm wax layer (melting T = 58–60°C) (Fluka, Madrid, Spain), preventing the complete mixing of PCR reactants until the reaction reached a temperature that minimizes the nonspecific annealing of primers to nontarget DNA. The lower solution contained the $MgCl_2$, the dNTPs, the primers, the $\alpha^{32}$P-dATP, and half of the buffer, and the upper solution contained the cDNA, the Taq enzyme, and the remaining buffer. PCR was performed for 28 cycles for CD4 and 23 cycles for HPRT, after 1 minute denaturation at 94°C. Each cycle consisted of denaturation at 92°C for 30 seconds, primer annealing at 57°C for CD4 or 60°C for HPRT for 1 minute, and primer extension at 72°C for 1 minute. Each PCR sample (5 $\mu$l) was electrophoresed in a 5% polyacrylamide gel. The gel was dried, and the radioactive bands were quantitated by phosphorimaging (Molecular Dynamics, Madrid, Spain). The sizes of the amplified products were 1073 bp for CD4 and 608 bp for HPRT.

### *ODN on proliferative response of primed T CD4$^+$ lymphocytes*

A group of animals were i.p. immunized with 0.1 mg/kg ovalbumin (OA, grade V) (Sigma) in Inject® Alum (Pierce, Rockford, IL). After 4 weeks, OA immunization was confirmed by specific antibody levels by ELISA (Salgado et al., 1988). Spleen lymphocytes were isolated as described. T cells were purified by passage through nylon wool, and T CD4$^+$ cells were negatively isolated with FITC-conjugated antirat CD8 mAb and

then passed through a MoFlo® Cell Sorter (DakoCytomation, Carpinteria, CA). T $CD4^+$ cells were ≥95%, as assessed by flow cytometry. Dendritic cells (DC) were obtained from unprimed rat spleen by Optiprep® gradient (Nycomed).

T $CD4^+$ cells ($1 \times 10^5$/well) were treated with ODN-C1, ODN-C4, AS-CD4-2, or AS-CD4-4 at optimal doses. After 24 hours, $1 \times 10^4$ DC treated with mitomycin C (50 $\mu$g/$5 \times 10^7$ DC) (Sigma) per well were added. T $CD4^+$ lymphocytes were subsequently stimulated with OA (1 $\mu$g/ml) or casein, as control protein, for 96 hours. In a parallel experiment, T $CD4^+$ cells were stimulated with concanavalin A (ConA) (Sigma) at 1 $\mu$g/ml for 48 hours. After incubation (37°C, 5% $CO_2$), the T $CD4^+$ cell proliferation was determined by a Cell Proliferation Biotrak ELISA system (Amersham) based on the measurement of 5′-bromo-2′-deoxyuridine (BrdU) incorporation into newly synthesized DNA (Porstmann et al., 1985). After reaction with peroxidase-conjugated anti-BrdU antibody and a specific substrate, absorbance was directly proportional to the amount of DNA synthesized.

*Statistical analysis*

Results were statistically analysed by the nonparametric U Mann-Whitney test. All analyses were performed using the Statistica computer package for Windows (Stat Soft, Tulsa, OK). Significance was set at $p < 0.05$.

## RESULTS

*Lymphocyte viability with AS-ODNs and DOTAP*

In previous studies, we have demonstrated by flow cytometry and confocal microscopy that DOTAP was essential to deliver AS-ODNs into rat lymphocytes (Rabanal et al., 2002). To determine the toxicity of DOTAP, lymphocytes were incubated with the cationic liposome at concentrations ranging between 5 and 20 $\mu$M and analysed after 4 and 24 hours (Fig. 1A). DOTAP at all the concentrations tested slightly reduced lymphocyte viability after 24 hours incubation. When ODN (0.1–5 $\mu$M) was added together with 10 $\mu$M DOTAP, no differences were observed between the concentrations assayed

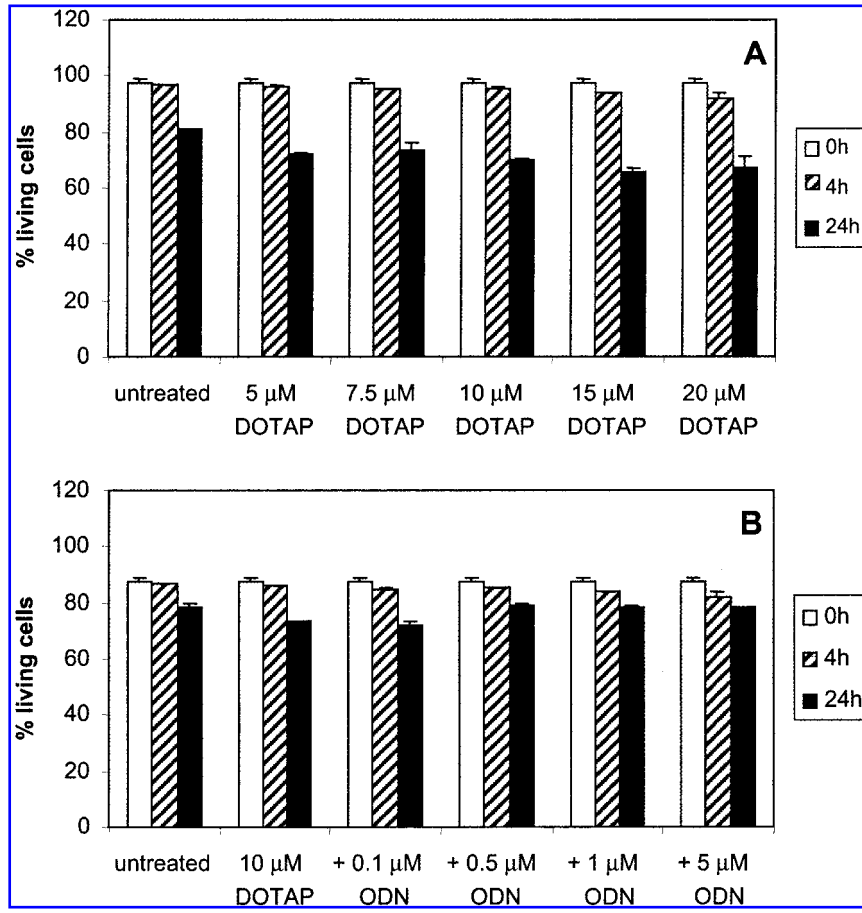


**FIG. 1.** (**A**) Effect of DOTAP concentrations (0–20 $\mu$M) on spleen lymphocyte viability. (**B**) Effect of several ODN-C1 concentrations (0.1–5 $\mu$M) with 10 $\mu$M DOTAP on spleen lymphocyte viability. Results are expressed as the percentage of living cells ($FDA^+IP^-$). Each point represents the mean of six values ± SEM.

(Fig. 1B). In any case, cell viability remained >70% after 24 hours.

### *Effect of AS-ODNs on CD4 surface expression after 24 hours of incubation*

CD4 expression was first studied in lymphocytes treated with the totally PS-modified AS-CD4-1 (0.25–4 $\mu$M) mixed with DOTAP. AS-CD4-1 did not affect expression compared with that determined in lymphocytes treated with unspecific ODN-C1 (data not shown). Thereafter, lymphocytes were incubated with the partially PS-modified AS-CD4-2, -3, and -4 mixed with DOTAP. Figure 2A shows the cytometry patterns corresponding to unlabeled lymphocytes (black peak) and lymphocytes treated either with DOTAP, DOTAP plus AS-CD4-2, or untreated, after labeling with anti-CD4-FITC mAb. Untreated labeled lymphocytes presented the highest MFI as the positive peak was placed on the right. Treatment with AS-CD4-2 displaced the positive peak toward the negative one, which reflected a decrease in MFI.

Results from lymphocytes incubated with increasing concentrations of the partially PS-modified AS-CD4-2, -3, and -4 (0.25–4 $\mu$M) mixed with a single concentration of DOTAP (7.5 $\mu$M) are summarized in Figure 2B. AS-CD4-2 and AS-CD4-4 inhibited CD4 surface expression by almost 40%. The effect was not dose dependent because the maximal effect was detected at 0.5 and 0.75 $\mu$M for both AS-CD4-2 and AS-CD4-4. These effects were significant compared with lymphocytes treated with DOTAP alone and lymphocytes treated with ODN-C2 and ODN-C4 ($p < 0.05$). AS-CD4-3 showed a significant effect only at 4 $\mu$M.

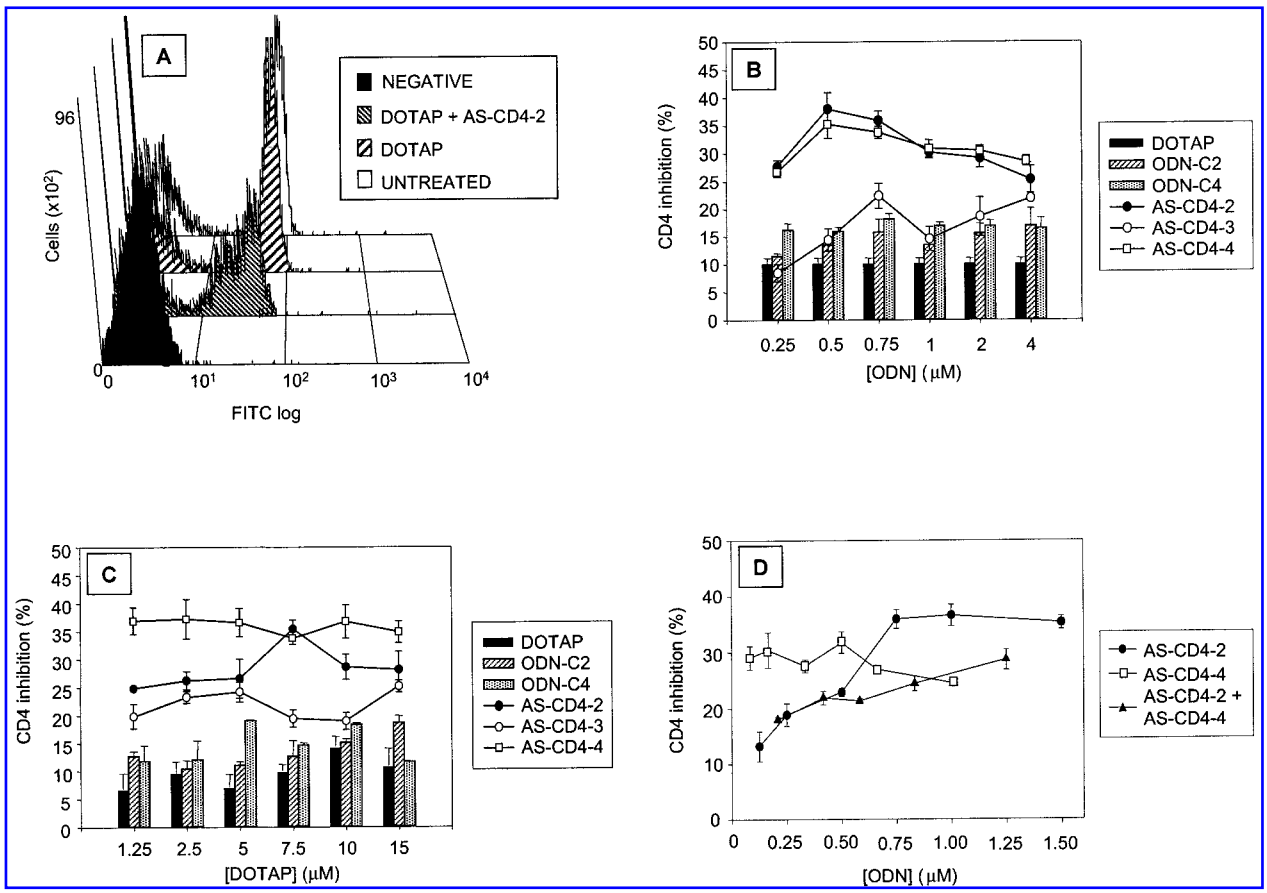


**FIG. 2.** Effect of AS-ODNs on cell surface CD4 expression. (**A**) Cytometry patterns of unlabeled lymphocytes (black peak) and lymphocytes treated with DOTAP, DOTAP plus AS-CD4-2, or untreated, after labeling with anti-CD4 mAb. (**B**) Effect of several concentrations of the partially PS-modified ODNs (0.25–4 $\mu$M) with DOTAP (7.5 $\mu$M). Results are expressed as the percentage of surface CD4 inhibition compared with that of untreated lymphocytes. (**C**) Effect of partially PS-modified ODNs (0.75 $\mu$M) mixed with several concentrations of DOTAP (1.25–15 $\mu$M). Results are expressed as percentage of surface CD4 inhibition compared with that of untreated lymphocytes. (**D**) Effect of increasing concentrations of two partially PS-modified ODNs together or separately. Results are expressed as the percentage of surface CD4 inhibition compared with untreated lymphocytes. Each point represents the mean of five values ± SEM.

The ODN/DOTAP ratio was subsequently analyzed using AS-CD4-2, -3, and -4 at 0.75 $\mu$M with several concentrations of DOTAP (1.25–15 $\mu$M). Lymphocytes treated with AS-CD4-2 showed the maximal inhibition of CD4 expression at 7.5 $\mu$M DOTAP (ODN/DOTAP ratio 1:10) (Fig. 2C). AS-CD4-4 exerted the same effect at any ratio studied. This inhibitory effect was significant compared with lymphocytes treated with DOTAP alone and lymphocytes treated with ODN-C2 and ODN-C4 ($p < 0.05$). Maximal CD4 inhibition in lymphocytes treated with AS-CD4-3 did not exceed 25% and was achieved at 15 $\mu$M DOTAP. In this condition, the AS-CD4-3 effect was significant compared with the lymphocytes treated with DOTAP and DOTAP plus ODN-C2 and ODN-C4 ($p < 0.05$).

The effectiveness of AS-CD4-2 and AS-CD4-4 was also tested by adding increasing concentrations of AS-ODNs mixed with DOTAP, keeping the ODN/DOTAP ratio at 1:10 for AS-CD4-2 and 1:15 for AS-CD4-4. The inhibitory effect of AS-CD4-4 remained around 30% and, again, did not show dose dependence (Fig. 2D). On the other hand, the dose-dependent response was quantified when AS-CD4-2 was added, reaching the maximal effect at 0.75 $\mu$M ODN plus 7.5 $\mu$M DOTAP. The effectiveness of AS-CD4-2 and AS-CD4-4 was also tested by adding both AS-ODNs together at concentrations ranging between 0.125 $\mu$M of AS-CD4-2 plus 0.083 $\mu$M AS-CD4-4 and 0.75 $\mu$M AS-CD4-2 plus 0.5 $\mu$M AS-CD4-4. Although a positive dose-response was found, no further inhibition was detected at any of the conditions assayed. Effects of increasing concentrations of AS-CD4-2 and AS-CD4-4, alone or combined, were significantly higher than that produced either by DOTAP alone or DOTAP plus ODN-C2 and ODN-C4 (data not shown).

### *Effect of AS-ODNs on other lymphocyte surface markers*

To assess the specificity of AS-ODNs, their effect on the surface expression of other lymphocyte molecules was studied. After 24 hours of addition of DOTAP alone (2.5, 5, and 7.5 $\mu$M) or DOTAP plus AS-CD4-2 or AS-CD4-4 (0.75 $\mu$M), lymphocyte CD3, CD5, and CD8 surface expression was analyzed by flow cytometry. Results were expressed as MFI (Table 2) and as the percentage of inhibition of surface molecule expression with respect to that

TABLE 2. EFFECT OF DOTAP ALONE OR COMBINED WITH AS-CD4-2 OR AS-CD4-4 IN LYMPHOCYTE SURFACE CD3, CD5, AND CD8 EXPRESSION[a]

| | | *MFI*[a] | | |
|---|---|---|---|---|
| | *DOTAP ($\mu$M)* | *CD3* | *CD5* | *CD8* |
| Untreated | — | 7.805 ± 0.16 (5) | 7.152 ± 0.18 (5) | 12.183 ± 0.19 (5) |
| Dotap alone | 2.5 | 7.332 ± 0.18 (5) | 7.003 ± 0.13 (5) | 13.240 ± 0.15 (5) |
| | 5 | 7.637 ± 0.11 (5) | 7.109 ± 0.17 (5) | 12.932 ± 0.25 (5) |
| | 7.5 | 7.487 ± 0.26 (5) | 6.942 ± 0.12 (5) | 13.064 ± 0.18 (5) |
| DOTAP + AS-CD4-2 ($0.75 \mu$M) | 2.5 | 8.325 ± 0.14 (5) | 7.359 ± 0.08 (5) | 13.719 ± 0.20 (5) |
| | 5 | 8.238 ± 0.24 (5) | 7.433 ± 0.07 (5) | 14.110 ± 0.31 (5) |
| | 7.5 | 8.063 ± 0.44 (5) | 7.314 ± 0.05 (5) | 13.620 ± 0.30 (5) |
| DOTAP + AS-CD4-4 ($0.75 \mu$M) | 2.5 | 7.894 ± 0.22 (5) | 6.950 ± 0.11 (5) | 12.840 ± 0.38 (5) |
| | 5 | 7.755 ± 0.17 (5) | 6.936 ± 0.06 (5) | 13.400 ± 0.51 (5) |
| | 7.5 | 7.725 ± 0.18 (5) | 6.734 ± 0.08 (5) | 13.821 ± 0.44 (5) |

Brackets ( ) indicate the number of samples.

[a]Results are expressed as mean fluorescence intensity (MFI) of positive cells after staining with FITC-conjugated specific mAb.

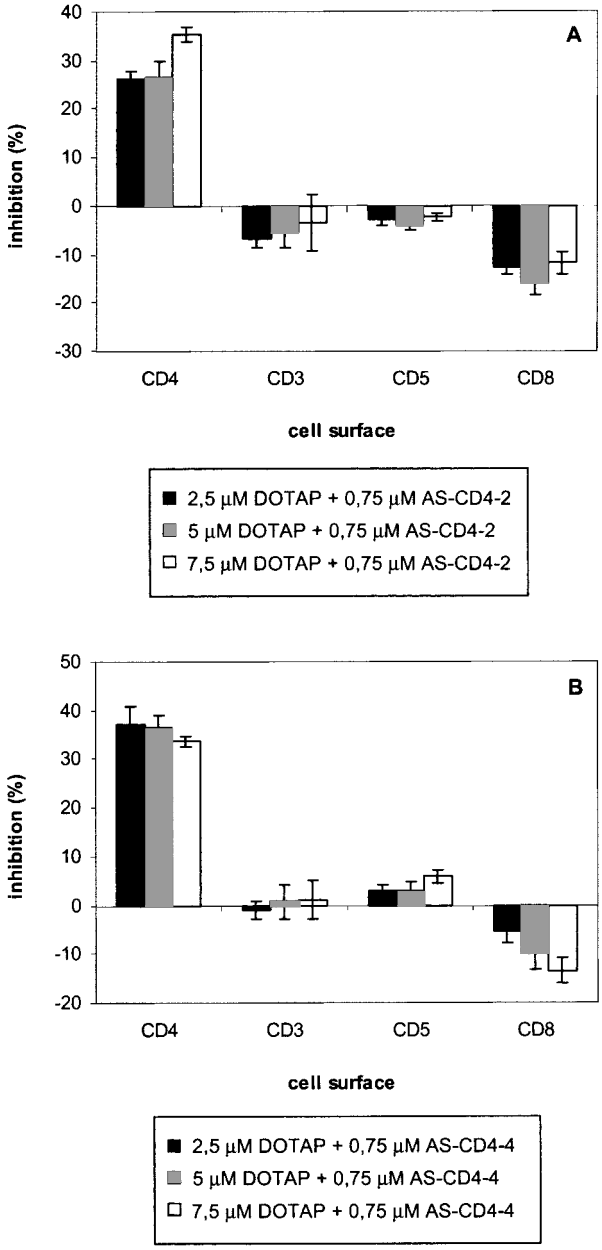


**FIG. 3.** Effect of the AS-ODNs on other cell surface molecules. (**A**) Effect of 0.75 $\mu$M AS-CD4-2 with several DOTAP concentrations (2.5–7.5 $\mu$M) on CD3, CD5, and CD8 expression. Results are expressed as the percentage of inhibition compared with that of untreated lymphocytes. (**B**) Effect of 0.75 $\mu$M AS-CD4-4 with several DOTAP concentrations (2.5–7.5 $\mu$M) on CD3, CD5, and CD8 expression. Results are expressed as the percentage of inhibition compared with untreated lymphocytes. Each point represents the mean of five values ± SEM.

determined for untreated lymphocytes. Neither AS-CD4-2 (Fig. 3A) nor AS-CD4-4 (Fig. 3B) was inhibitory in the three cell surface molecules studied. No significant differences were observed between MFI of lymphocytes treated with DOTAP alone and MFI of lymphocytes treated with AS-CD4-2 or AS-CD4-4 in CD3, CD5, or CD8 expression.

### *Effect of AS-ODNs on surface CD4 expression after 72 hours*

Lymphocyte CD4 surface expression after treatment with a single addition of the partially PS-modified AS-ODNs was evaluated after 3 days (Fig. 4). The inhibitory effect of AS-CD4-2 and AS-CD4-4 remained for at least 72 hours ($p < 0.05$ compared with lymphocytes treated with DOTAP alone or treated with ODN-C2 and ODN-C4). The effect of AS-CD4-3 also remained for 72 hours. Unexpectedly, the nonspecific effect caused by ODN-C2 and ODN-C4 disappeared after 48 hours. Therefore, the effect of specific AS-ODN was clearer at 2–3 days of treatment, when the unspecific effect disappeared.

### *Effect of AS-CD4-2 and AS-CD4-4 on lymphocyte CD4 mRNA expression*

To test whether AS-CD4-2 and AS-CD4-4 downregulated lymphocyte mRNA, CD4 mRNA expression was quantified by RT-PCR after 24 hours and 48 hours of AS-ODN incubation. After 24 hours, the treatment with AS-CD4-2 and AS-CD4-4 decreased CD4 mRNA expression up to 37% and 42%, respectively (Fig. 5). At 48 hours, CD4 mRNA decreased up to 27% for AS-CD4-2 and 29% for AS-CD4-4. Therefore, the action of AS-CD4-2 and AS-CD4-4 might be through activation of RNAse H. In addition, CD4 mRNA expression was quantified after ODN-C1 incubation, and the inhibitory effect was about 5%–10% (data not shown).

### *Effect of AS-ODNs on proliferative response of T CD4$^+$ lymphocytes*

The proliferative response to specific and unspecific stimuli of T CD4$^+$ lymphocytes treated with AS-CD4-2, AS-CD4-4, ODN-C1, and ODN-C4 is summarized in Figure 6. Inhibition of surface CD4 expression by AS-CD4-2 and AS-CD4-4 was confirmed before stimulus addition. Proliferation of primed T CD4$^+$ lymphocytes induced by OA was almost totally inhibited by treatment with AS-CD4-2 and AS-CD4-4 ($p < 0.05$ with respect to untreated or ODN-C1, ODN-C4-treated lymphocytes) (Fig. 6A). The proliferative response to ConA of AS-ODN-treated lymphocytes showed that AS-CD4-2 and AS-CD4-4 partially inhibited this proliferation ($p < 0.05$ with respect to untreated or ODN-C1, ODN-C4-treated lymphocytes) (Fig. 6B).

## DISCUSSION

Complementary and antisense nucleic acids have been used to regulate gene activity for 20 years. As a result, several clinical studies focus on the use of AS-ODNs as therapeutic drugs. PS-modified oligonucleotides are currently being evaluated in clinical trials for their ability to

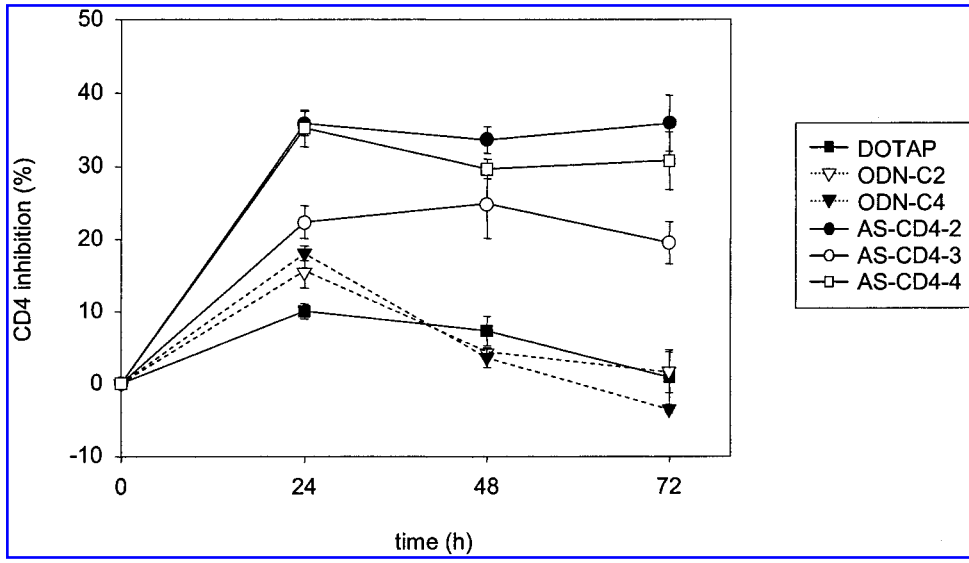


**FIG. 4.** Time course of the effect of ODNs on lymphocyte CD4 surface expression for 3 days. Each point represents the mean of six values ± SEM.

inhibit restenosis (reviewed in Santiago and Khachigian, 2001) and various tumors (reviewed in Wang et al., 2001). Recently, the first antisense drug, Vitravene® (Isis Pharmaceuticals, Carlsbad, CA), was approved for commercialization by the FDA, and several other antisense drugs have shown promising activities in clinical trials.

The CD4 membrane glycoprotein, present in Th lym-

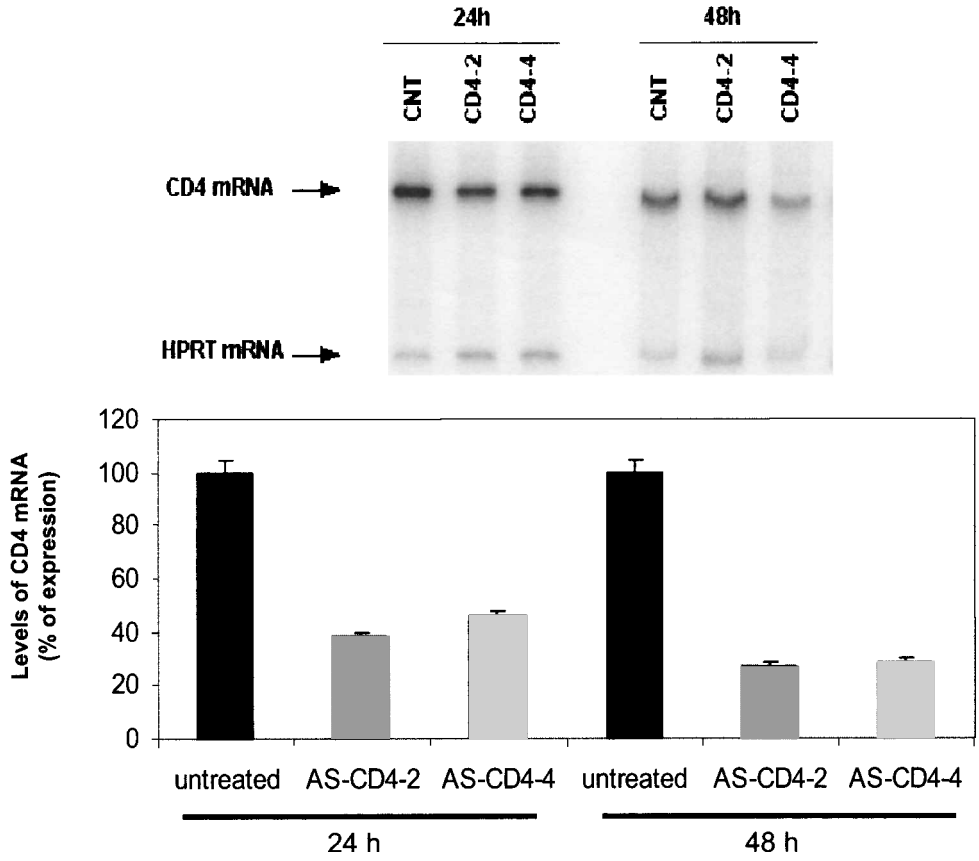


**FIG. 5.** Effect of AS-CD4-2 and AS-CD4-4 on CD4 mRNA expression. HPRT mRNA was used as internal control. (**Top**) Representative autoradiography of the PCR products. (**Bottom**) Quantitation of the bands. Each point represents the mean of three values ± SEM.

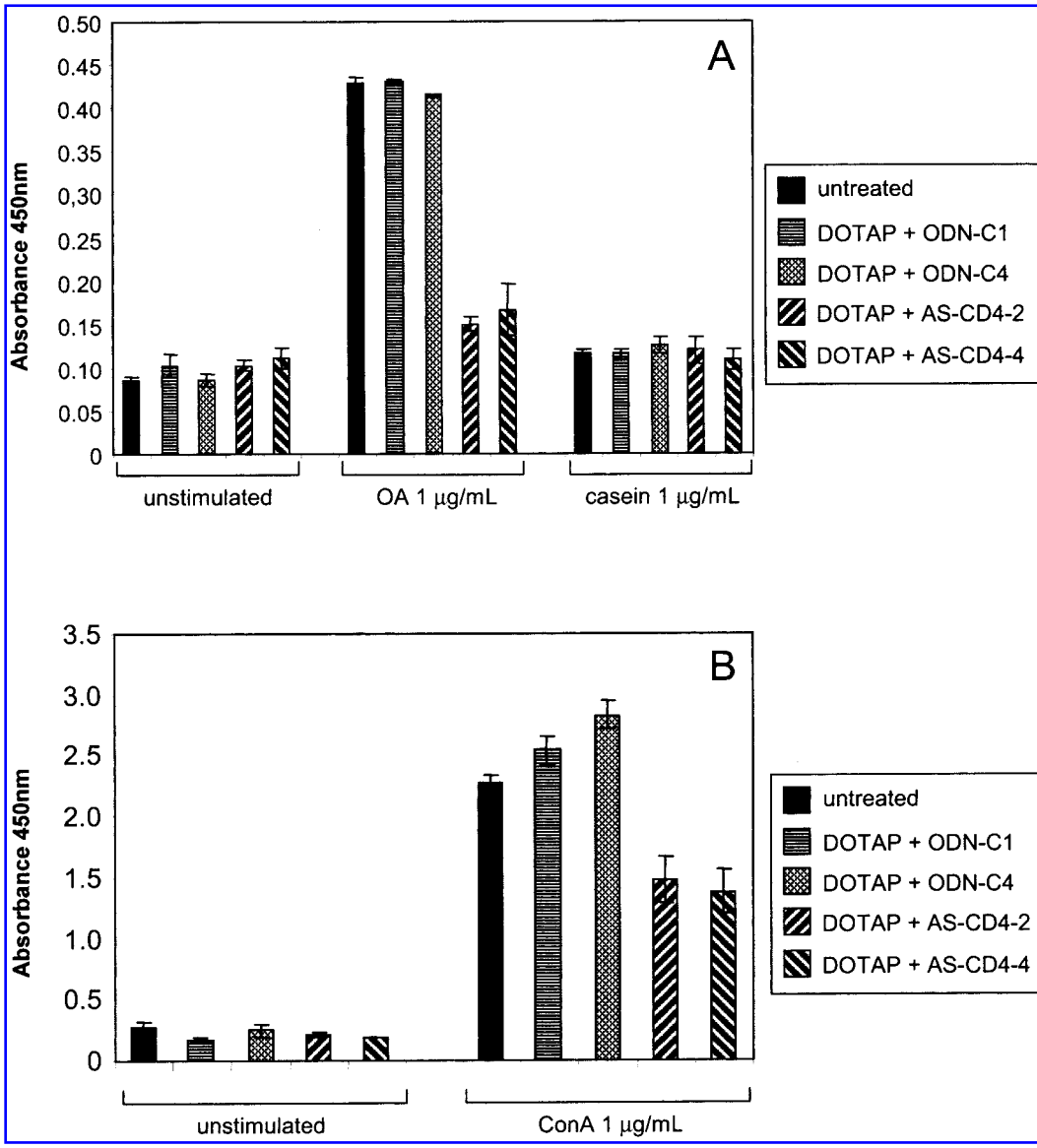


**FIG. 6.** Effect of AS-ODN on the proliferative response of OA-primed T CD4$^+$ lymphocytes. (**A**) BrdU incorporation after OA-specific and unrelated protein (casein) stimuli. (**B**) BrdU incorporation after ConA stimulus. Each point represents the mean of four values ± SEM.

phocytes, is essential to the immune response because it stabilizes the physical interactions between T cells and APCs and increases the intracellular signaling activities triggered by antigen interaction with specific T cell receptor (Janeway, 1989; Cammarota et al., 1992). Inhibition of CD4 surface expression induces immune response modulation. Thus, anti-CD4 mAb therapy prevents and ameliorates rat adjuvant arthritis (Pelegrí et al., 1995, 1996). Moreover, it has been described that anti-CD4 mAb efficiently induces immune tolerance in the treatment of human and other types of experimental arthritis (Mauri et al., 1997; Isaacs et al., 1999; reviewed in Nepom, 2002; reviewed in Keystone, 2002) and in the prevention of transplantation rejection (Mottram et al., 1998; Motoyama et al., 2000; reviewed in Adler and Turka, 2002). However, mAb treatment can induce an antiantibody response, rendering the treatment eventually ineffective (Pelegrí et al., 1996).

Surface CD4 synthesis stimulated by PMA can be reduced by partially PS-modified AS-ODNs directed against *cd4* mRNA (Rabanal et al., 2002). Here, we have demonstrated that freshly isolated spleen lymphocytes treated with the same partially PS-modified AS-ODNs decreased CD4 surface expression. The AS-ODNs specifically inhibited CD4 and did not affect CD3, CD5, or CD8 molecules. The AS-ODN effect remained for at least 72 hours. The cells used in the present study were untreated rat spleen lymphocytes obtained early in the morning. Rat CD4 surface expression follows a circadian rhythm, with a daily variation of 17% (Pelegrí et al.,

2003). The minimal number of CD4 surface molecules is reached at the end of the active period (i.e., at the end of the dark period in nocturnal animals), which coincides with the maximal amount of blood Th lymphocytes. Therefore, although the rat Th lymphocytes used in this study were not PMA-treated, the time of the day when they were collected and treated corresponded to the minimal CD4 expression, from which a certain increase in CD4 surface expression was expected.

The administration of totally PS-modified AS-ODN decreased CD4 surface expression, but this effect was unspecific, as reported for PMA-treated lymphocytes with the same AS-ODN (Rabanal et al., 2002). Therefore, it appears that fully PS-modified AS-ODNs produce unspecific effects on the surface CD4 expression of both primary and PMA-treated rat lymphocytes. Taylor et al. (1996) described an unspecific increase in tumor necrosis factor-$\alpha$ (TNF-$\alpha$) release after incubation of macrophages with fully PS-modified AS-ODN against TNF. AS-CD4-2, with the same sequence but a distinct degree of PS modification from AS-CD4-1, according to Peyman and Uhlmann (1996), showed a specific effect. This different effect may be attributed to an increase in efficiency and specificity to the hybridization to the endogenous RNA when using minimal modifications. On the other hand, CpG-containing ODNs can present immunostimulatory properties that are more marked in PS-ODNs (reviewed in Krieg, 1999).

Two of the three partially PS-modified AS-ODNs effectively decreased both surface and mRNA CD4 expression. All the AS-ODNs tested were directed against the translation start site of the rat *cd4* gene, but the effective AS-ODN had eight bases in common between the 5′-UTR and the AUG start codon. AS-ODNs may arrest RNA translation by binding to the translational initiation codon and the 5′-UTR (Crooke, 1998, 1999). However, their effectiveness may be conditioned to other characteristics, such as mRNA sequences and predicted secondary structures, which may be associated with oligonucleotide binding. In this regard, Yun et al. (2000) examined the inhibition of lymphocyte IL-2 secretion by AS-ODNs, and only one of the three AS-ODNs directed against the translation start codon displayed the desired effect. Moreover, Boeve and De Ley (1994) studied seven AS-ODNs against the translation initiation codon of the human interferon-$\gamma$ (IFN-$\gamma$) mRNA, and only one caused a significant inhibition. Therefore, the AS-ODNs directed against the mRNA of lymphocytes were not always effective. However, other types of AS-ODNs directed against lymphocyte mRNA inhibit some phases of immune responses. Harel-Bellan et al. (1988) showed that as AS-ODN against IL-2 inhibits the proliferation of the Th1 clone but has no effect on Th2 clones. AS-ODNs against IL-4 showed the opposed effect. Furthermore, AS-ODNs effectively target lymphocyte surface CD80 and CD86 and thus prevent acute rejection allografts (Qian et al., 2001; Liang et al., 2001). AS-ODNs directed against cell surface molecules offer great potential for anti-inflammatory strategies. AS-ODNs targeting intercellular adhesion molecule-1 (ICAM-1) have been used in inflammatory bowel disease and for the suppression of allograft rejection (Stepkowski et al., 1998; Yacyshyn et al., 1998; Van Assche and Rutgeerts, 2002). The results of the phase I trial of ISIS 104838, a 2′-methoxyethyl-modified AS-ODN targeting TNF-$\alpha$1, have recently been published (Sewell et al., 2002). The control of the secreted IgM (excessive production or autoantibody) of B lymphocytes by AS-ODNs has also been achieved in a number of disorders, such as rheumatoid arthritis and systemic lupus erythematosus (McCall, 1998).

Our data point to the efficacy of AS-CD4-2 and AS-CD4-4 in downregulating mRNA expression. It appears that AS-ODNs, by hybridizing to the target mRNA, stimulate RNAse H, which degrades the RNA strand of RNA-DNA duplexes (Phillips and Gyurko, 1995). The exact recognition elements for RNAse H are unknown. The effect of AS-CD4-2 and AS-CD4-4 remained for 72 hours, which was the longest time lymphocytes survived without stimulation. These data support that AS-ODNs are powerful immunomodulating agents against CD4. We have established that mAb therapy against CD4 required doses applied at 48-hour intervals (Caballero et al., 1998). Thus, although the effect of AS-ODNs on CD4 *in vivo* remains to be elucidated, it may be a long-term treatment.

To assess whether the decrease in CD4 expression induced by ODN was enough to block T $CD4^+$ reactivity, specific and unspecific proliferation assays were performed. T $CD4^+$ lymphocytes obtained from OA-primed rats and treated with a single dose of the effective AS-ODNs, AS-CD4-2 and AS-CD4-4, showed a lower reactivity to polyclonal mitogen ConA. Moreover, the effective single dose of AS-CD4-2 or AS-CD4-4 almost totally inhibited the specific reactivity of T $CD4^+$ lymphocytes after 4 days. These results show that although surface and mRNA CD4 expression was not fully abolished by AS-ODN at 72 hours, it was enough to block T $CD4^+$ reactivity. In summary, we have found two AS-ODN sequences (AS-CD4-2 and AS-CD4-4) directed against the translation start region of *cd4* that, interacting with mRNA, reduced surface CD4 expression and almost totally inhibited the antigen-specific immune response. These results demonstrate the immunomodulatory power of a single dose of AS-ODN and, therefore, its potential as a therapeutic strategy in autoimmune diseases.

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Address reprint requests to:
*Dr. Margarida Castell*
*Departament de Fisiologia–Divisió IV*
*Facultat de Farmàcia*
*Av. Joan XXIII s/n*
*E-08028 Barcelona*
*Spain*

*E-mail:* margac@farmacia.far.ub.es

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