

Modulation of T-cell function by (R)- and (S)-isomers of albuterol: Anti-inflammatory influences of (R)-isomers are negated in the presence of the (S)-isomer

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Background: β_2 -Adrenergic agonists interact with specific receptors on T lymphocytes to mediate anti-inflammatory activities. However, anti-inflammatory effects are not observed when β -adrenergic agonists are administered in vivo as racemates. **Objective:** We hypothesized that anti-inflammatory influences are mediated by the (R)-isomer and are masked in the additional presence of the (S)-isomer.

Methods: Antigen-specific T-cell lines were generated in the presence of recombinant human IL-2 and tetanus with or without varying concentrations of (R)- and (S)-isomers of albuterol alone or in combination. Parallel lines were generated in the presence of propranolol. Cells were briefly pulsed with PHA and evaluated for proliferation, apoptosis, and cytokine secretion.

Results: (R)-Albuterol significantly inhibited T-cell proliferation ($77.0\% \pm 9.7\%$ of control at 10^{-8} mol/L and $61.1\% \pm 9.0\%$ at 10^{-7} mol/L). No influence was observed with (S)-albuterol alone. However, the addition of (S)-albuterol to (R)-albuterol mediated a dose-dependent increase in proliferation. At equivalent concentrations of the 2 isomers, proliferation was unchanged from the control, whereas at 10^{-6} mol/L (S)-albuterol, proliferation was enhanced. Both the inhibitory effects of (R)-albuterol alone and the stimulating influence of (R)- plus (S)-albuterol were blocked in the additional presence of propranolol. (R)-Albuterol at 10^{-8} mol/L inhibited IL-2 and IFN- γ production. Racemic albuterol (10^{-8} mol/L each) had no influence on cytokine production; however, the combination of 10^{-8} mol/L (R)-albuterol with 10^{-6} (S)-albuterol stimulated production of IL-2 and IL-13. No effects were observed on apoptosis or cell viability.

Conclusion: These studies confirm the β -adrenergic receptor-specific anti-inflammatory effects of (R)-albuterol. The racemate had minimal influences on proliferation or cytokine production. The presence of excess (S)-albuterol

resulted in proinflammatory influences. We hypothesize that the (S)-isomer functions as an inverse agonist to switch the function of the β_2 -adrenergic receptor. (J Allergy Clin Immunol 2002;109:449-54.)

Key words: Albuterol, β -adrenergic receptor, racemic, T lymphocyte, cytokine, apoptosis, proliferation

The inappropriate and persistent activation of CD4⁺ T_H lymphocytes produces the characteristic inflammatory phenotype of asthma. T cell-derived cytokines activate eosinophils, increase mast cell production and the tendency to degranulate, induce the IgE isotype switch, and promote inflammation. IL-4 and other cytokines further contribute to the persistence of these inflammatory processes by inhibiting T-cell activation-induced cell death (apoptosis) and promoting resistance to the anti-inflammatory influences of corticosteroids. Although many cells, including eosinophils and mast cells, may produce these cytokines, the initial antigen-driven phase of this cascade is a T-cell process. Inhibition of T-lymphocyte function is one of the more important biologic activities of the primary class of drugs used to treat asthma, corticosteroids, and underlies the clinical efficacy of immunotherapy. These observations support the importance of identifying therapeutic agents that modulate T-lymphocyte function and thereby lessen allergic inflammation. T lymphocytes possess surface β -adrenergic receptors of the β_2 -subtype.¹ Although T lymphocytes have a low density of β -receptors, these receptors are in a high-affinity state.² Engagement of β_2 -receptors on T cells mediates diminished proliferation and cytokine production.³⁻⁷ Engagement of β_2 -receptors should thereby mediate anti-inflammatory effects. However, despite rigorous investigations, anti-inflammatory influences of β_2 -agonists have never been shown in vivo, and some studies have suggested that β_2 -agonists may paradoxically worsen asthma control by increasing airway inflammation and bronchial hyperreactivity.⁸⁻¹¹

Most currently available β_2 -agonists are racemic mixtures composed of a 50:50 mixture of (R)- and (S)-isomers. Clinical studies with single-isomer (R)-albuterol have demonstrated that, when given in amounts equivalent to that found in the racemate, greater bronchodilation was achieved. When half that amount was given, bronchodilation was equivalent to that achieved with the

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Abbreviation used

cAMP: Cyclic adenosine monophosphate
PI: Propidium iodide

racemate and was associated with fewer side effects.^{12,13} (S)-Albuterol binds to the adrenergic receptor with 100-fold less affinity than (R)-albuterol but does not function as a bronchodilator.¹⁴ (S)-Albuterol induces paradoxical reactions in airways and may decrease asthma control.^{10,15,16} Furthermore, the metabolic clearance of (S)-albuterol is slower than that of (R)-albuterol. The resultant accumulation of (S)-albuterol could contribute to these potential adverse effects on asthma control.¹⁷⁻¹⁹

We therefore hypothesized that the (S)-isomers of β_2 -agonists may be biologically active and have either proinflammatory actions or otherwise mask the expected anti-inflammatory effects of the (R)-isomers when administered as part of a racemic mixture. Thus we compared the effects of the individual isomers and their combination on T-cell proliferation, cytokine production, and apoptosis on antigen-specific T-cell lines.

METHODS

Reagents

(R)- and (S)-Albuterol were obtained from Sepracor, Inc. (Marlborough, Mass.).

Generation of antigen-specific T-cell lines

Tetanus-specific lines were prepared according to the methodology of Agarwal and Marshall.²⁰ Heparinized venous blood was obtained from healthy human volunteers (18-60 years old) by using a protocol approved by the Human Investigation Committee at the University of Virginia. PBMCs were isolated through Ficoll-Hypaque (Sigma, St Louis, Mo) density gradient centrifugation. The cells were washed and resuspended in complete RPMI-1640 medium (Life Technologies, Rockville, Md) containing 1 mol/L HEPES (Life Technologies) and 10,000 U/mL penicillin and 10 μ g/mL streptomycin (Life Technologies) supplemented with 10% autologous serum. Freshly prepared PBMCs (1×10^6 cells/mL in 5 mL) were cultured in 6-well tissue-culture plates with or without the additional presence of pharmacologically relevant concentrations of (R)-albuterol (10^{-8} – 10^{-7} mol/L) and (S)-albuterol (10^{-9} – 10^{-6} mol/L), alone or in various combinations. Parallel samples were cultured in the presence of 10 μ mol/L propranolol (Wyeth-Ayerst Laboratories, St Davids, Pa). After preincubation, 0.5 mg/mL tetanus toxoid (Wyeth Laboratories) and recombinant human IL-2 (16 pg/mL; Pharmingen, San Jose, Calif) were added to each culture. The cell cultures were incubated in a 5% CO₂ humidified environment at 37°C for 2 to 6 days. At the end of this incubation period, each culture was briefly pulsed with a submitogenic concentration of PHA (0.5 mg/mL, Sigma) to maximize cytokine secretion, and after a further 24-hour incubation, supernatants were collected and frozen for later analysis by means of ELISA. The cell pellets were washed and analyzed for apoptosis and flow cytometry, as described below.

Proliferation assays

On days 2, 4, and 6, 2×10^5 cells were removed from the 6-well tissue-culture plates and plated in quadruplicate on 96-well plates. The following day, cells were pulsed with 50 μ Ci of tritiated thymidine

(ICN, Aurora, Ohio) and incubated overnight. Proliferation responses were assayed by using a FilterMate 196 harvester (Packard, Downers Grove, Ill) and thymidine incorporation values were determined on a TopCount-NXT (Packard) microplate scintillation counter.

Cytokine determination

IL-2, IL-5, IL-13, and IFN- γ were measured in cell-culture supernatants with commercial ELISA kits (BioSource, Camarillo, Calif), according to the manufacturer's instructions. ELISAs were performed on an EL808 ultra microplate ELISA reader (Bio-Tek, Winooski, Vt). Concentrations were calculated on the basis of standard curves by using the KC4 Kineticalc for Windows software (Bio-Tek). The sensitivities of these assays were less than 16 pg/mL for IL-2, less than 4 pg/mL for IL-5, less than 12 pg/mL for IL-13, and less than 4 pg/mL for IFN- γ .

Apoptosis

Cell pellets obtained from the day-6 cultures were washed and exposed to 5 μ g of FITC-conjugated Annexin V and 2 μ L of propidium iodide (PI; Sigma). Fluorescence was analyzed on a FAC-Scalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) by using the CellQuest software. Cells committed to apoptosis were quantified by means of their surface binding of annexin V, and cell viability was determined by their exclusion of PI. Data were analyzed as the percentage of viable cells and as the percentage of annexin V-positive cells in both the PI-positive and PI-negative populations. Cell-surface expression of apoptosis and cell-activation markers were evaluated by using FITC-conjugated CD4 and 2 μ g of R-phycoerythrin-conjugated CD28, CTLA-4, Bcl-2, and Fas (Pharmingen, San Diego, Calif). For the staining with Bcl-2, lymphocytes were permeabilized through exposure to 0.2% Tween-20 (Sigma) for 15 minutes. The flow cytometer was gated to collect data only on CD4⁺ T_H lymphocytes, and data were analyzed as both the percentage of cells expressing each marker and as mean fluorescent intensity.

Statistical analysis

All data are displayed as means $\pm$ SEM. Statistical analyses were performed with the use of Friedman nonparametric repeated measures on a Gateway E-4200 Pentium III processor with JMP3.0 software.

RESULTS

Proliferation: (R)-albuterol

Proliferation in the presence of (R)-albuterol was reduced to $77.0\% \pm 9.7\%$ of control lymphocytes at 10^{-8} mol/L ($P < .001$) and to $61.1\% \pm 9.0\%$ at 10^{-7} mol/L ($P < .001$; the mean $\pm$ SEM of 6 replicate results is shown in Fig 1). These effects were mediated by β -adrenergic receptors, as shown by the loss of antiproliferative effects in the additional presence of the β -adrenergic receptor antagonist propranolol (Fig 2). We next determined whether this antiproliferative effect of (R)-albuterol was the result of the shifting of the time course of maximal proliferation of these antigen-specific lines. Evaluation of tritiated thymidine incorporation on days 2, 4, and 6 demonstrated no change in the proliferation response curve (data not shown). Maximum inhibition of proliferation was observed at day 6; however, smaller influences were observed at each of the previous time points in a time-dependent fashion. (R)-Albuterol (10^{-8} – 10^{-6} mol/L) mediated a dose-dependent inhibition of proliferation, with maximum inhibition observed at 10^{-6} mol/L (data not shown).

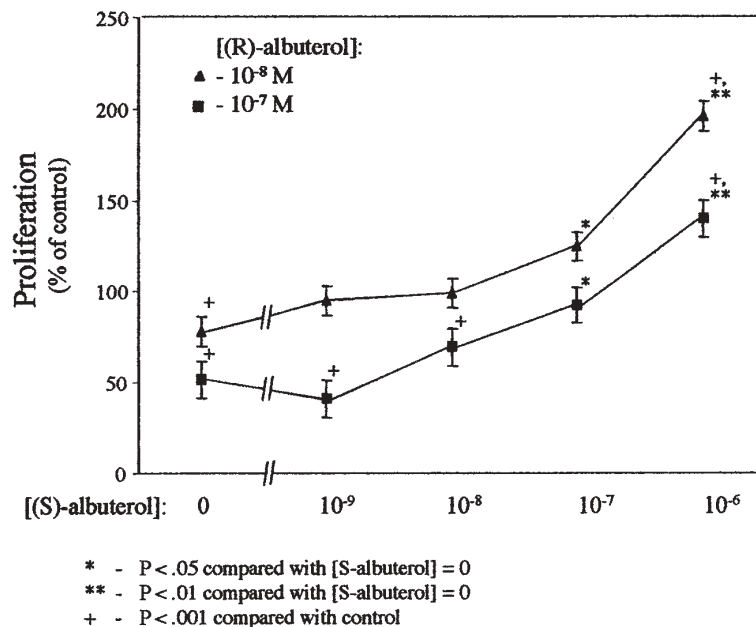


FIG 1. Modulation of T-lymphocyte proliferation by albuterol. Antigen-specific T-cell lines were generated by culturing PBMCs with tetanus toxoid. Cells were cultured with and without the additional presence of (R)-albuterol (10^{-8} - 10^{-7} mol/L) and (S)-albuterol (10^{-9} - 10^{-6} mol/L) alone or in combination. Proliferation was assayed as tritiated thymidine incorporation, and data are presented as the percentage of the proliferation obtained without albuterol.

TABLE I. Influence of (R)- and (S)-albuterol and cytokine secretion

Cytokine	Control	(R)-Albuterol, 10 ⁻⁸ mol/L	(R)-Albuterol, 10 ⁻⁸ mol/L + (S)- albuterol, 10 ⁻⁸ mol/L	(R)-Albuterol, 10 ⁻⁸ mol/L + (S)- albuterol, 10 ⁻⁶ mol/L	(R)-Albuterol, 10 ⁻⁷ mol/L	(R)-Albuterol, 10 ⁻⁷ mol/L + (S)- albuterol, 10 ⁻⁷ mol/L
IL-2	1070 ± 253	716 ± 50*	1415 ± 101	3530 ± 288*	549 ± 53*	1573 ± 86*
IFN- γ	145 ± 29	85 ± 9*	106 ± 11	160 ± 10	65 ± 10*	154 ± 7
IL-13	126 ± 15	98 ± 7	145 ± 4	183 ± 14	89 ± 7	126 ± 8
IL-5	254 ± 52	158 ± 34	185 ± 24	244 ± 40	102 ± 38*	239 ± 15

Cytokine concentrations are given in picograms per milliliter.

* $P < .05$ compared with control.

Proliferation: (S)-albuterol

No influence on proliferation was observed with (S)-albuterol alone (10^{-8} - 10^{-7} mol/L, data not shown). The addition of (S)-albuterol to (R)-albuterol mediated dose-dependent increases in proliferation (Fig 1). At equivalent concentrations of the 2 isomers, as would be provided by racemic albuterol, proliferation was unchanged from that of the control lines. The additional presence of 10^{-6} mol/L (S)-albuterol to samples containing either 10^{-8} or 10^{-7} mol/L (R)-albuterol significantly stimulated proliferation. This stimulation of proliferation was mediated through the β -adrenergic receptor, as shown by its reversal in the additional presence of propranolol (data for 10^{-8} mol/L (R)-albuterol are displayed in Fig 2).

Cytokine production

(R)-Albuterol at 10^{-8} mol/L significantly inhibited IL-2 and IFN- γ secretion and, at 10^{-7} mol/L, inhibited pro-

duction of all 4 cytokines measured (Table I and Fig 3). By itself, (S)-albuterol did not influence cytokine production (data not shown). However, (S)-albuterol abrogated these inhibitory effects of (R)-albuterol when the isomers were administered as an equimolar racemate. In fact, at 10^{-7} mol/L, an equimolar combination of (R)- and (S)-albuterol significantly stimulated IL-2 production (Table I). The combination of 10^{-6} mol/L (S)-albuterol to 10^{-8} mol/L (R)-albuterol stimulated production of IL-2 and IL-13 (Table I and Fig 3).

Apoptosis

No effects were observed on apoptosis (Annexin V expression) or cell viability (PI exclusion) with any of the β_2 -adrenergic compounds. Annexin V expression was not modulated in either viable (PI-negative) or nonviable (PI-positive) populations. β -Adrenergic agents did not influence other cellular markers of apoptosis or cell activation, including Fas, Bcl-2, CD28, or CTLA-4.

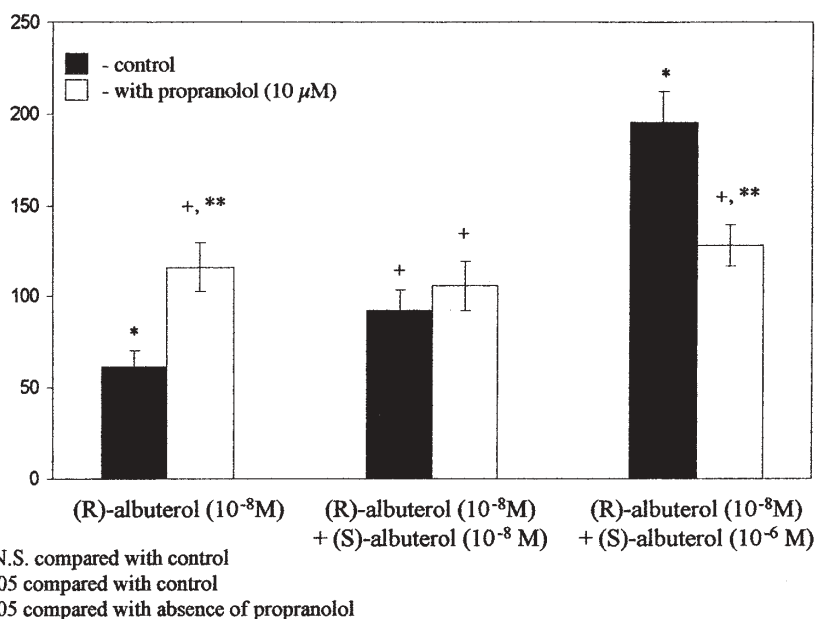


FIG 2. Effect of propranolol on T-lymphocyte proliferation modulated by (R)- and (S)-albuterol. Tetanus-specific T-cell lines were cultured with (R)-albuterol (10^{-8} mol/L) with or without (S)-albuterol (10^{-8} - 10^{-6} mol/L). Parallel samples were cultured in the additional presence of propranolol ($10 \mu\text{mol/L}$). Data are shown as percentages of the proliferation obtained without albuterol.

DISCUSSION

β_2 -Adrenergic agonists are among a large number of pharmaceutical agents that are administered as racemic mixtures. The (R)-isomer binds to β_2 -adrenergic receptors with greater affinity than the (S)-isomer (approximately 100-fold higher in the case of albuterol) and is responsible for the bronchodilation seen with the racemate.¹⁴ The (S)-isomer was presumed to be physiologically inert. However, the ability to separate the isomers of β_2 -adrenergic agents has raised doubts regarding this assumption. When studied *in vitro*, β_2 -agonists and other agents that elevate intracellular cyclic adenosine monophosphate (cAMP) mediate reduced cytokine gene transcription in T lymphocytes and monocytes and inhibit activation of neutrophils, eosinophils, and mast cells. However, efforts to document these anti-inflammatory influences *in vivo* have been disappointing,^{21,22} raising the possibility that these effects may be masked by the presence of the (S)-isomer. In support of this hypothesis, (S)-albuterol has been shown to have the potential for procontractile and proinflammatory effects. For example, (R)-albuterol produced the expected dose-dependent decrease in intracellular free Ca^{2+} concentration in bovine tracheal smooth muscle cells, whereas (S)-albuterol increased intracellular free Ca^{2+} .^{23,24} Proallergic influences of (S)-isomers of β -adrenergic agents have been shown on eosinophils and mast cells.^{25,26} (S)-Albuterol stimulated eosinophil recruitment and degranulation in contrast to (R)-albuterol, which inhibited these reactions.²⁶⁻²⁸ In ovalbumin-challenged guinea pigs reduced eosinophilia is observed with (R)-albuterol, but not (S)-albuterol, or racemic albuterol.²⁸

The clinical trials of (R)-albuterol (levalbuterol) in adults¹² and children¹³ demonstrated that this single-

isomer agent had superior clinical efficacy compared with the racemic mixture. That is, 1.25 mg of levalbuterol produced superior bronchodilation to that of 2.5 mg of the racemate (1.25 mg of each isomer) and was associated with a longer duration of action.¹² At 0.63 mg, levalbuterol produced efficacy comparable with that of 2.5 mg of the racemate.¹² Similarly, levalbuterol at one quarter the dose had comparable efficacy to the racemic mixture in protecting against exercise-induced bronchospasm²⁹ and, in comparison with the racemate, did not induce tolerance to the bronchoprotective effect against methacholine¹⁰ or cold-air³⁰ challenges. These data suggest that the presence of (S)-albuterol in the racemic mix may mask, in part, the clinical efficacy of the active bronchodilator.

Our studies demonstrate dramatic inhibition of T-lymphocyte proliferation and cytokine production by (R)-albuterol (Fig 1 and Table I). We can not exclude the possibility that (R)-albuterol may not directly influence T lymphocytes but instead acts in this assay to modulate accessory cell function. Although IL-2 was present in the culture media used to generate tetanus-specific T-cell lines, the concentration applied (16 pg/mL) was well below that measured in culture supernatants (Table I), suggesting that most of our measured IL-2 was freshly generated.

The concept that (S)-albuterol may have clinically harmful effects has been further supported by studies of subjects administered (R)-albuterol in the emergency department³¹ and in the hospital.³² These urgent-care settings may exaggerate the harmful effects of the (S)-isomer insofar as this agent has a longer pharmacologic half-life than the (R)-isomer and the frequent dosing of β_2 -adrenergic agents will therefore promote the accumulation of the (S)-isomer.¹⁹ The effect of frequent dosing

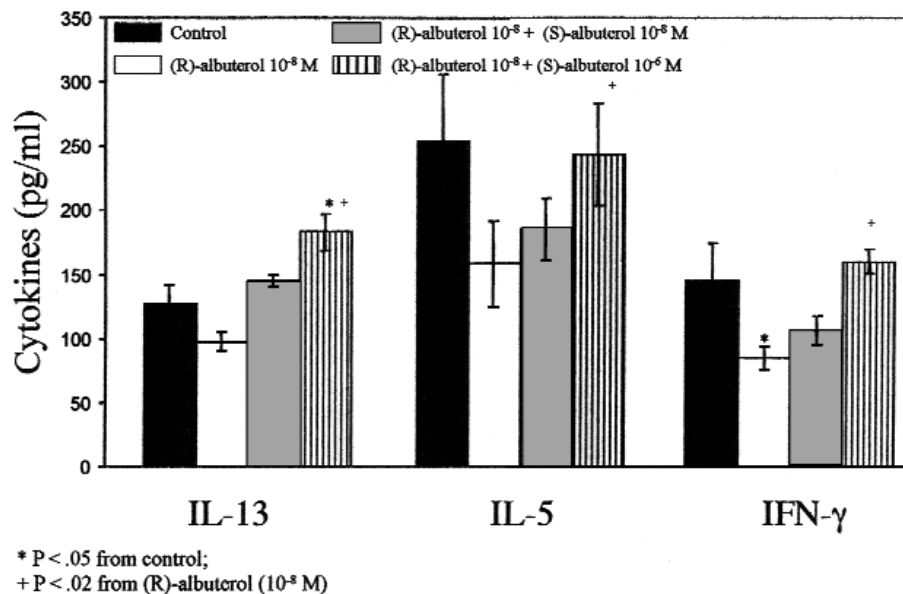


FIG 3. Modulation of cytokine production by albuterol. Tetanus-specific T-cell lines were cultured in the additional presence of (R)-albuterol (10⁻⁸ mol/L) with or without (S)-albuterol (10⁻⁸ or 10⁻⁶ mol/L). After 6 days, supernatants were collected and assayed by means of ELISA for IL-5, IL-13, and IFN-γ. Data are expressed as the concentration of cytokine in picograms per milliliter of supernatant.

of racemic albuterol would be to reproduce our experimental conditions in which lymphocytes were exposed to (R)-albuterol in the concomitant presence of higher concentrations of (S)-albuterol, conditions associated with prominent proinflammatory effects (Fig 1 and Table I).

The increase in proliferation observed with the combination of (S)-albuterol at 10⁻⁶ mol/L with (R)-albuterol (10⁻⁷-10⁻⁸ mol/L) surprisingly was inhibited in the additional presence of propranolol (Figs 1 and 2). These data demonstrate that this effect was mediated through β-adrenergic receptors and not through an independent effect of the (S)-isomer. Several mechanisms may be proposed for such a phenomenon. Inverse agonists are ligands that preferentially stabilize G protein-coupled receptors, such as the β-adrenergic receptor, in their inactive conformation.³³⁻³⁵ The (S)-isomer of albuterol may function as such an inverse agonist and by maintaining the β₂-adrenergic receptor in its inactive conformation could thereby act to reverse the result of β₂-adrenergic receptor-ligand binding (ie, lower cAMP of less than the basal level). Thus the (R)-isomer alone would trigger increased intracellular cAMP and anti-inflammatory effects, whereas in the additional presence of the (S)-isomer, decreased intracellular cAMP and enhanced proliferation would be observed. Alternatively, agonist-promoted activation of the β₂-receptor results in receptor coupling with G_i proteins in a protein kinase A-dependent fashion.³⁶ Should (S)-albuterol demonstrate enhanced affinity for the G_i-coupled receptor, this would explain the proinflammatory effects of the isomer, which would ultimately be mediated through MAP kinase pathways.

Our results on cytokine production are consistent with many, but not all, previous studies investigating influences of racemic β-adrenergic agents on cytokine pro-

duction by T lymphocytes *in vitro*.^{3,5,6,37} and are consistent with known influences of cAMP-elevating agents on T-cell cytokine production.^{4,7,38-41} Several researchers reported decreased cytokine production after exposure of T lymphocytes to the β-agonists.^{3,5,6} These studies, as well as ours, demonstrate inhibition of both T_H1- and T_H2-associated cytokines by β-adrenergic agents. However, other studies on the influences of β-agonists on cytokine production by T cells have produced conflicting results. Our data contrast those found by Agarwal and Marshall,²⁰ which showed a T_H1-to-T_H2 shift mediated by albuterol, with decreased IFN-γ but enhanced IL-4 and IL-5 production. Panina-Bordignon et al⁴² and Oh (personal communication) also reported a paradoxical increase in IL-4 production. These conflicting results may reflect experimental differences, including choice of antigen and the use of PBMCs in contrast to antigen-specific T-cell lines. Antigens may differ in their inherent propensity to promote T_H1- or T_H2-like responses that may supercede influences mediated by the β-adrenergic agents. We used tetanus toxoid in our studies because it is an antigen to which subjects are routinely immunized, it is associated with a relatively high precursor frequency in the circulation, and it readily generates both T_H1- and T_H2-like responses, regardless of the atopic predisposition of the subject. One final explanation for differences observed in cytokine production may reflect the use of racemic mixtures in earlier studies that would have been confounded by influences of the (S)-isomer. For example, in contrast to results seen in control cells, cell lines exposed to 10⁻⁸ mol/L (R)-albuterol had decreased IFN-γ production with the concomitant addition of an equimolar concentration of (S)-albuterol (Table I). However, when higher concentrations of (S)-albuterol were

applied (10^{-6} mol/L), we observed a significant increase in IL-13 production. These results are reminiscent of the T_H1 -to- T_H2 shift described in the work of Agarwal and Marshall²⁰ and are probably consistent with the variability inherent in these assays.

In summary, these studies demonstrate anti-inflammatory influences of albuterol on antigen-specific T-cell lines, including inhibition of proliferation and cytokine production. These effects were limited to the (R)-isomer of albuterol and were eliminated in the additional presence of the (S)-isomer. Previous *in vivo* studies with racemic mixtures have been unable to demonstrate inhibition of inflammation by β -adrenergic agents and have suggested that their prolonged use may paradoxically worsen asthma control. Further studies are warranted to demonstrate whether removal of the (S)-isomer will lead to the demonstration of anti-inflammatory influences of β -adrenergic agents *in vivo*.

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