In Vitro and In Vivo Effects of Leukotriene B$_4$ Antagonism in a Primate Model of Asthma

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Abstract

To test the hypothesis that leukotriene (LT) B$_4$ antagonists may be clinically useful in the treatment of asthma, CP-105,696 was evaluated in vitro, using chemotaxis and flow cytometry assays, and in vivo, using a primate asthma model. CP-105,696 inhibited LTB$_4$-mediated monkey neutrophil chemotaxis (isolated cells, LTB$_4$ = 5 nM) and CD11b upregulation (whole blood, LTB$_4$ = 100 nM) with IC$_{50}$ values of 20 nM and 16.5 μM, respectively. Using a modification of a previously described in vivo protocol (Turner et al. *Am. J. Respir. Crit. Care Med.* 1994. 149: 1153–1159), we observed that treatment with CP-105,696 inhibited the acute increase in bronchoalveolar lavage (BAL) levels of IL-6 and IL-8 by 56.9±13.2% and 46.9±14.5%, respectively, 4 h after challenge with *Ascaris suum* antigen (Ag). CP-105,696 tended to reduce the increase in BAL protein levels 0.5 h after Ag challenge by 47.5±18.3%, but this was not statistically significant. In addition, CP-105,696 prevented the significant 11-fold increase in airway responsiveness to methacholine after multiple Ag challenge. These results suggest that LTB$_4$ partially mediates acute and chronic responses to antigen in an experimental primate asthma model and support the clinical evaluation of LTB$_4$ antagonists in human asthma. (*J. Clin. Invest.* 1996. 97:381–387.) Key words: CP-105,696 • leukotriene B$_4$ • hyperresponsiveness • asthma • primates

Introduction

Leukotriene (LT)[1] B$_4$ is derived from the metabolism of arachidonic acid by the enzyme, 5-lipoxygenase (1). This potent, proinflammatory lipid mediator is produced by many cells including neutrophils, monocytes, macrophages, keratinocytes, lymphocytes, and mast cells (2). In guinea pigs (3) and dogs (4), LTB$_4$ administration causes marked pulmonary infiltration of neutrophils and eosinophils as well as an increase in airway responsiveness to bronchospastic agonists such as histamine or acetylcholine.

Because these responses are commonly observed in asthma (5), the antagonism of LTB$_4$ may be useful therapeutically for the treatment of this condition. To test this hypothesis, we used a nonhuman primate model of asthma. In this model, animals (*Macaca fascicularis*) demonstrate a statistically significant increase in BAL protein levels, which is indicative of vascular leak, 0.5 h after exposure to a single antigen challenge. Four hours post challenge, they demonstrate increases in BAL neutrophils and peak increases in cytokines (6). Upon repeated antigen challenge on alternate days, these monkeys develop airway hyperresponsiveness (AHR) to methacholine (MCh), which is accompanied by a marked pulmonary eosinophil influx (7). Thus, this animal model exhibits many of the same responses that are observed in asthmatic patients and provides a biological system in which the effects of LTB$_4$ inhibition can be evaluated.

In the current study, LTB$_4$ inhibition was achieved using CP-105,696, a potent (IC$_{50}$ = 8.42±0.26 nM in an [^{3}H] LTB$_4$ human neutrophil binding assay) and selective antagonist of LTB$_4$ (8) that also inhibits LTB$_4$-mediated human neutrophil chemotaxis (5.0±2.0 nM) and LTB$_4$-mediated upregulation of CD11b in human neutrophils (pA$_2$ = 8.03±0.19) (8). This is a structurally novel compound that has demonstrated oral activity in several in vivo guinea pig and murine models of inflammation (9). In the current study, we evaluated this compound's ability to inhibit nonhuman primate neutrophil chemotaxis and CD11b upregulation and, having confirmed similar potency in both primate and human neutrophil assays, evaluated CP-105,696 for its ability to prevent in vivo inflammation and the development of AHR.

Methods

Monkey neutrophil isolation and chemotaxis assay. Neutrophils were isolated from anticoagulated (EDTA) monkey blood according to Rot (10). Isolated neutrophils were resuspended (2.5 × 10^6 cells/ml) in HBSS (supplemented with 10 mM Hepes) containing 0.7 mM Mg^{2+} and 1.6 mM Ca^{2+} and 2 mg/ml recrystallized bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) and adjusted to pH 7.25. The chemotaxis assay was performed in a 48-well chamber apparatus (Neuroprobe, Cabin John, MD) using cellulose nitrate filters (pore size 3.0 mm) as described previously (11). The total number of cells (observed at 400×) migrating from 20 mm from beneath the surface monolayer to the leading front (usually ∼ 100–120 mm per 60 min at optimal chemotactic factor concentrations) in response to varying concentrations of chemotactic factors were summed with the aid of an Optimax image analyzer (Optimax, Hollis, NH) and provided an index of the chemotactic response. Each experimental condition was performed in duplicate and three to four fields were assessed for cell migration. Results are expressed as the percentage maximum response where 100% was equal to the peak response seen in the presence of the most efficacious concentration of chemotactic factor. The number of cells migrating spontaneously (i.e., negative controls) were subtracted from all measurements prior to data transformation. In ex-

1. *Abbreviations used in this paper:* Ag, *Ascaris suum*; AHR, airway hyperresponsiveness; BAL, broncho alveolar lavage; LT, leukotriene; MCF, mean channel flourescence; MCh, methacholine.

periments where CP-105,696 was evaluated for its ability to block chemotaxis, equal concentrations were present in both compartments of the chemotaxis chamber.

Neutrophil CD11b upregulation assay. Monkey blood was collected by venipuncture into vacutainer tubes containing EDTA. The whole blood was incubated for 5 min at 37°C either in the presence of or absence of the indicated concentration of CP-105,696. Aliquots (100 µl) of whole blood + CP-105,696 were then added to the tubes containing 10 µl LTB$_4$ 10× stock and were further incubated for 10 min at 37°C. The samples were placed into an ice water bath and 1 ml of cold phosphate buffered saline (PBS) containing 10 mM EDTA, pH 7.25, 0.2% sodium azide and 2% heat inactivated fetal bovine serum (PBS-wash). The cells were pelleted by gentle centrifugation (200–250 g) at 4°C, the supernatant was removed by aspiration, and the samples were resuspended in the remaining fluid by gentle shaking. The cells were incubated with saturating concentrations of fluorescein-conjugated anti-CD11b (Bear 1 clone; Gen Trak Inc.) for 30 min at 4°C. To control for nonspecific antibody: cell interactions, samples were incubated with FITC-conjugated murine IgG1 of irrelevant epitope. To each sample, 1 ml PBS-wash was added and the cells were pelleted as before. Red blood cells were lysed with Becton Dickinson (Mountain View, CA) FACS® Lysing Solution as per manufacturer's directions. After two washings in PBS-wash, the cells were resuspended in 0.5–1 ml PBS-wash and the degree of fluorescence was determined on a Becton Dickinson FACscan® flow cytometer in conjunction with a Hewlett Packard computer with a Consort 32 program. Data was collected in the list mode and the neutrophil gates were defined by the forward scatter/side scatter dot plots. Neutrophil staining was quantified by histogram analysis and expressed as mean channel fluorescence (MCF).

Single Ag challenge procedure. The first in vivo study was performed using six atopic monkeys to ascertain the effects of LTB$_4$ inhibition on acute Ag-induced responses. Blood was sampled at time 0 for baseline measurements. A dose of 30 mg/kg CP-105,696 was orally administered to three monkeys. Three monkeys received vehicle only. Blood samples were obtained 16 and 20 h after drug administration for assessment of plasma drug concentrations. BAL was performed 16h after drug administration using one 15-ml wash of PBS. Each monkey was then challenged with *Ascaris suum* antigen (Ag)

aerosol using a concentration previously determined to double respiratory system resistance, R$_{rs}$. A second BAL was performed as before (on opposite side of lung) for leukocyte count 4 h after Ag administration (20 h post compound). 2 wk later (because of the extremely long half life of the drug in this species, $t_{1/2}$ = 53 h), the experiment was repeated with control and treated monkeys reversed. In this study, we measured the acute increase in BAL total and differential leukocyte number in control and treated monkeys. Total cell counts were determined using a Cell-Dyn 3500 Cell Counter (Abbott, Chicago, IL). To determine the percent composition of each leukocyte type, two slides from each monkey BAL sample were obtained by centrifuging 2 × 150 µl of the lavage fluid for 2 min at 500 rpm in a Cytospin centrifuge (Shandon Instruments, Pittsburgh, PA). The slides were stained in Diff-Quick for differential cell count. Two hundred leukocytes were counted on each slide. The percent composition of each cell type was averaged for the two slides.

Multiple Ag challenge (airway hyperresponsiveness) procedure. Seven atopic, anesthetized (10 mg/kg Ketamine, 1.0 mg/kg Rompun, IM) and intubated monkeys were used in each experiment. The pre-Ag PC$_{200}$ to methacholine (MCh) aerosol was determined on day 0 by delivering increasing concentrations of the agonist (30 breaths, 1 min) via a Devilbis ultrasonic nebulizer connected, in series, to a Harvard ventilator. Lung function was monitored over 40 cycles for each MCh concentration until the provocative concentration that doubled baseline resistance (PC$_{200}$) was obtained. Rrs was calculated by taking the mean respiratory system impedance, measured by discrete-frequency sinusoidal forced oscillations superimposed upon tidal breathing, for the entire frequency range to obtain a single R$_{rs}$ value as described previously (12).

After MCh concentration-response measurements were complete, the endotracheal tube was detached from the circuit, and an Olympus fiberoptic bronchoscope was inserted through the endotracheal tube and wedged into a 3rd to 5th generation bronchus. Three 15-ml aliquots of saline were injected into the lung and gently aspirated. The pooled BAL sample was analyzed for total and differential leukocytes as previously described. Blood samples were drawn on days 0 and 10 for hematology measurements.

On days 2 through 9, the same monkeys were orally administered 10 mg/kg CP-105,696 which, in previous pharmacokinetic studies,
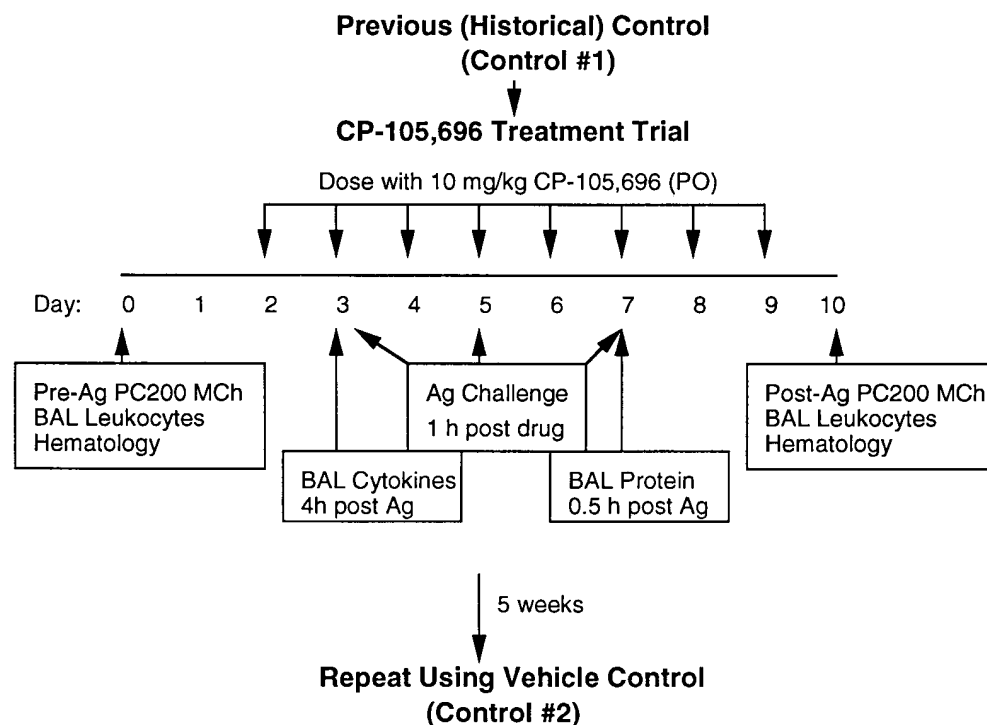

Previous (Historical) Control (Control #1)

CP-105,696 Treatment Trial

Dose with 10 mg/kg CP-105,696 (PO)

Day: 0 1 2 3 4 5 6 7 8 9 10

Pre-Ag PC200 MCh
BAL Leukocytes
Hematology

Ag Challenge
1 h post drug

Post-Ag PC200 MCh
BAL Leukocytes
Hematology

BAL Cytokines
4h post Ag

BAL Protein
0.5 h post Ag

5 weeks

Repeat Using Vehicle Control (Control #2)

Figure 1. Protocol used for multiple antigen challenge study in CP-105,696-treated monkeys and for bracket controls (control 2).

produced efficacious plasma levels for 24 h. On day 3, the monkeys were anesthetized and intubated as described previously. The monkeys then received Ag aerosol in a concentration predicted to double their R_{rs} based on previous experiments. 4 h after Ag challenge, a BAL was performed as previously described on the opposite side of the lung using 15 ml of PBS. The BAL samples were centrifuged for 10 min at 3,000 rpm. The supernatant was collected and concentrated to 2 ml in Centriprep-3 concentrators (Amicon, Inc., Beverly, MA) and analyzed for IL-1β, IL-6, IL-8, and GM-CSF using commercially available ELISA kits (R & D Systems, Minneapolis, MN).

On day 5, the Ag challenge protocol described for day 3 was repeated except that no lavages were performed. R_{rs} was measured to confirm that the concentration of Ag in use actually caused airway resistance to double. On day 7, the Ag challenge protocol described for day 3 was repeated, but this time BAL was performed 0.5 h after Ag challenge to determine total protein levels in the BAL fluid. Based on previous studies, this time point is when the peak protein leakage is observed in this model. BAL fluid was concentrated as previously described and analyzed for total protein using a commercially available kit (Sigma Chemical Co., St. Louis, MO). On day 10, the procedures outlined for day 0 were repeated to obtain the post Ag MCh PC_{200}, total and differential leukocyte counts in the BAL fluid and in the peripheral blood.

Five to six weeks after each monkey had completed the CP-105,696 treatment protocol, an identical bracket control experiment was performed except that monkeys received vehicle sham treatment instead of CP-105,696 on days 2–9.

Using the above procedure, every attempt was made to ensure that BAL was performed in a different airway so that no individual location was lavaged more than once during the course of each 10-d study. Blood samples were drawn for determination of plasma levels of CP-105,696 at the time of Ag challenge. Blood and BAL fluid was analyzed for urea content to allow determination of the dilution effect of lavage as previously described (13). This protocol is summarized in Fig. 1.

There was no significant difference in the volume of BAL fluid recovered on any day of the study. Furthermore, control and CP-105,696 treatment groups did not significantly differ in the volume of BAL fluid obtained from each.

Statistics were performed using Statview 4.01 (Abacus Concepts, Berkeley, CA). Acute Ag challenge experiments were analyzed using a paired t test. Data from the multiple Ag challenge procedure were analyzed using a one way ANOVA to compare historical controls (control 1), treated monkeys and bracket controls (control 2). Historical controls represent the most recent control experiment in which each monkey participated prior to the CP-105,696 treatment study. In all experiments, each monkey served as its own control. A Bonferroni-Dunnett's post hoc test was performed on the data to determine which means were statistically different, as indicated by a P value < 0.05.

Animal care and use procedures were performed with the authorization of the Animal Care and Use Committee at Pfizer Central Research and are in accordance with the principles outlined by the Declaration of Helsinki.

Results

A dose of 30 mg/kg (PO) was used in the single Ag study and plasma levels of 61.3 ± 5.6 µg/ml were obtained 16 h after dosing, immediately prior to Ag challenge. In the multiple Ag challenge study, a dose of 10 mg/kg CP-105,696 was administered. The day 3 sample was collected 24 h after the first dose (29.3 ± 2.5 µg/ml), the day 5 sample was collected after 3 d of dosing (50.8 ± 2.7 µg/ml) and the day 7 sample was collected after 5 d of dosing (56.1 ± 3.1 µg/ml). All samples were obtained immediately prior to Ag challenge. The results indicate that there was ample systemic exposure to the compound at the time of antigen challenge in both studies which is consistent with the half-life of this drug.

In vitro studies demonstrated that LTB_4 stimulates the chemotaxis of monkey neutrophils with the maximum effect observed at $\sim$ 10 nM (see insert, Fig. 2). At a constant LTB_4
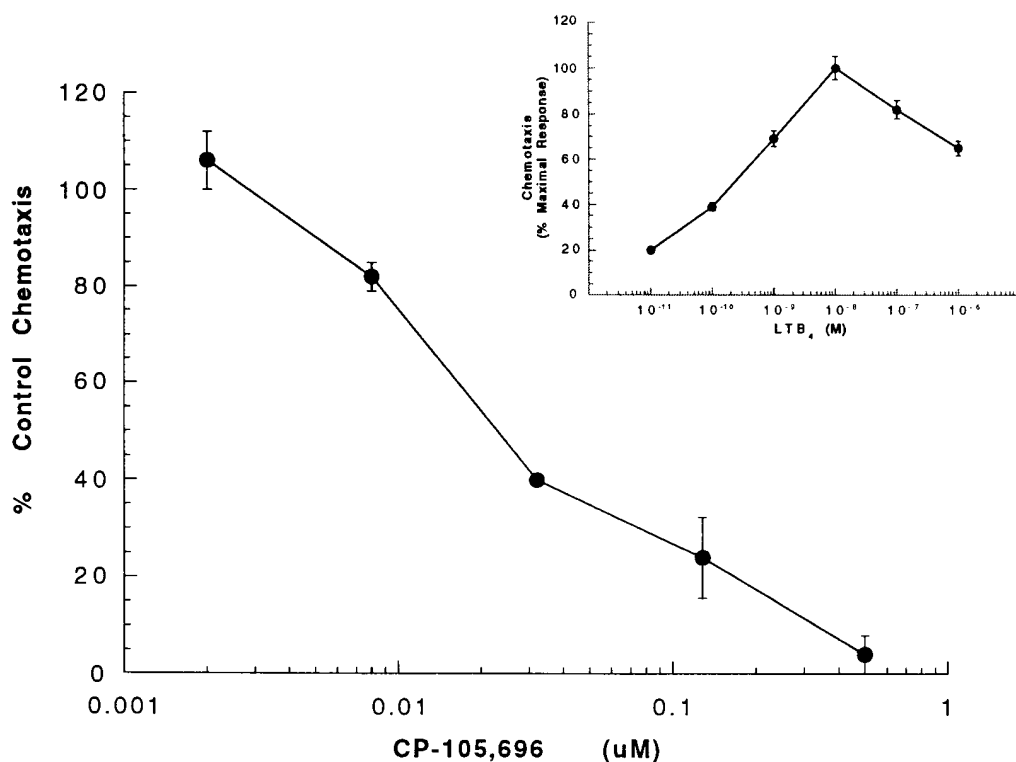

Figure 2. Inhibition of LTB_4-mediated chemotaxis of monkey neutrophils by CP-105,696. LTB_4 was used at a fixed concentration of 5 nM in the lower chamber, and CP-105,696 was present in varying concentrations in the upper and lower chambers. Chambers were incubated for 60 min at 37°C, and then the filters were removed, processed and counted as described in Methods. Data are means$\pm$SEM from 3 to 5 independent experiments. Insert shows the LTB_4 concentration-response curve.
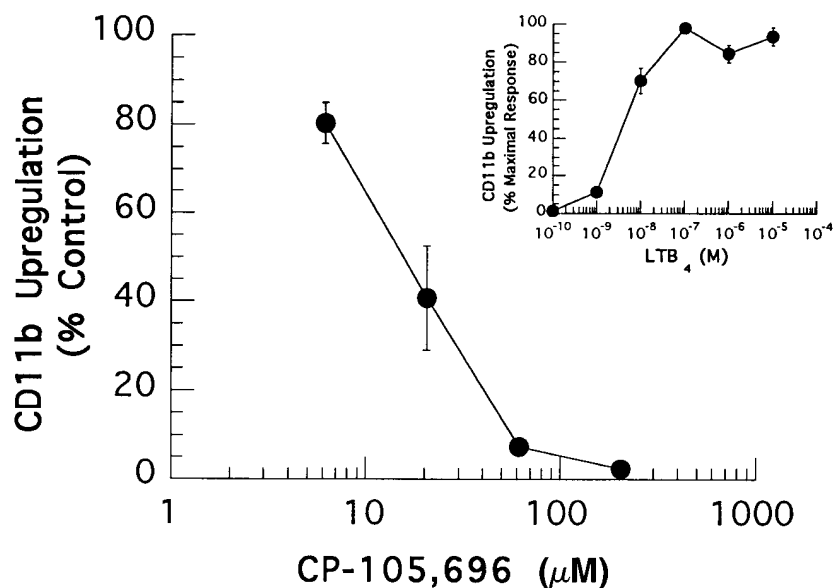
Figure 3. Inhibition of LTB$_4$-mediated CD11b upregulation on neutrophils in monkey whole blood. LTB$_4$ was used at a fixed concentration of 100 nM. CP-105,696 was present in varying concentrations (0.5–200 μM). Whole blood neutrophils were incubated for 10 min at 37°C, and then samples were processed for FACS analysis as described in Methods. Data are expressed as the percentage of the response occurring in the absence of CP-105,696 and are mean±SEM from four to five independent experiments. Insert shows LTB$_4$ concentration-response curve.

concentration of 5 nM, CP-105,696 inhibited LTB$_4$-mediated monkey neutrophil chemotaxis in a dose-dependent manner with an IC$_{50}$ of 0.02±0.001 μM (Fig. 2). Furthermore, LTB$_4$ also stimulated the upregulation of CD11b on monkey neutrophils in whole blood in a concentration-dependent manner with an EC$_{50}$ of 5.07±1.22 nM (see insert, Fig. 3). At a constant LTB$_4$ concentration of 100 nM, CP-105,696 inhibited CD11b upregulation in a dose-dependent manner with an IC$_{50}$ of 16.50±4.24 μM (Fig. 3).

In the single Ag challenge experiment (Fig. 4), there was no significant difference in the baseline BAL neutrophil number of control (3.69±2.64 × 10^3 neutrophils/ml) and treated (1.90±1.07 × 10^3 neutrophils/ml) monkeys. Ag challenge caused a significant 85.5±36.7-fold increase in neutrophils 4 h post challenge of controls (2.42±0.61 × 10^5 neutrophils/ml). CP-105,696 significantly reduced ($P = 0.043$) the increase in BAL neutrophils to 33.5±8.81-fold (6.67±3.51 × 10^4 neutrophils/ml) 4 h post Ag challenge. No other leukocyte type was significantly elevated at this time point.

As part of the 10 day AHR protocol, the levels of BAL cytokines were determined 4 h after the first Ag challenge on day 3 (Fig. 5). As previously observed, exposure to Ag results in significant increases in IL-1β, IL-6, IL-8, and GM-CSF. Compared to the control trial in the same monkeys, CP-105,696 caused a significant reduction in BAL levels of IL-6 (control: 2715±663 pg/ml vs. CP-105,696: 1187±598 pg/ml; $P = 0.048$) and IL-8 (control: 956±248 pg/ml vs. CP-105,696: 540±158 pg/ml; $P = 0.022$).

The levels of BAL protein in monkeys treated with CP-105,696 were compared with those obtained on day 7 of their control AHR trial. Although there was a tendency for BAL protein levels to be decreased in the CP-105,696-treated group (6.88±3.66 mg/ml), compared to controls (16.14±4.99 mg/ml), this was not a statistically significant reduction ($P = 0.070$).

The remaining results from this study include end points that reflect the responses to multiple Ag challenge. In Fig. 6, the MCh PC$_{200}$ values for day 0 and day 10, i.e., before and after multiple Ag challenge, are presented for both control and treatment trials. In both control trials, multiple Ag challenge caused a significant decrease in the MCh PC$_{200}$ (Control# 1:

0.90±0.32 mg/ml (day 0) to 0.15±0.09 mg/ml (day 10); Control# 2: 7.14±5.65 mg/ml (day 0) to 0.61±0.51 mg/ml (day 10)). In monkeys treated with CP-105,696, the MCh PC$_{200}$ did not significantly differ on day 0 (1.21±0.65 mg/ml) and day 10 (2.43±1.83 mg/ml). Thus, CP-105,696 was protective against Ag-induced decreases in MCh PC$_{200}$, our indicator of AHR. Note: only five monkeys are included for this particular end-point because two monkeys bronchoconstricted to PBS thus, precluding measurement of their MCh PC$_{200}$.
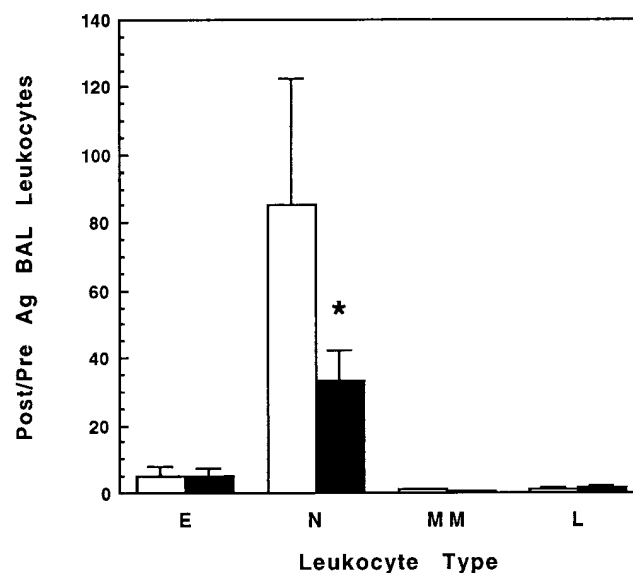

Figure 4. The mean ratio of post: pre Ag BAL leukocytes (±SEM) 4 h after a single challenge with *Ascaris suum* antigen in six monkeys when treated with vehicle only (*open bars*) and the same six monkeys treated with 30 mg/kg CP-105,696 (*closed bars*) each monkey serving as its own control in a cross-over experimental design. In this study, Ag challenge caused a significant increase in the number of BAL neutrophils. This was significantly reduced by CP-105,696 pre treatment ($P < 0.05$*). No other cell type was significantly elevated by Ag challenge at this time point. (*E*, eosinophils; *N*, neutrophils; *MM*, monocytes/macrophages; *L*, lymphocytes.)
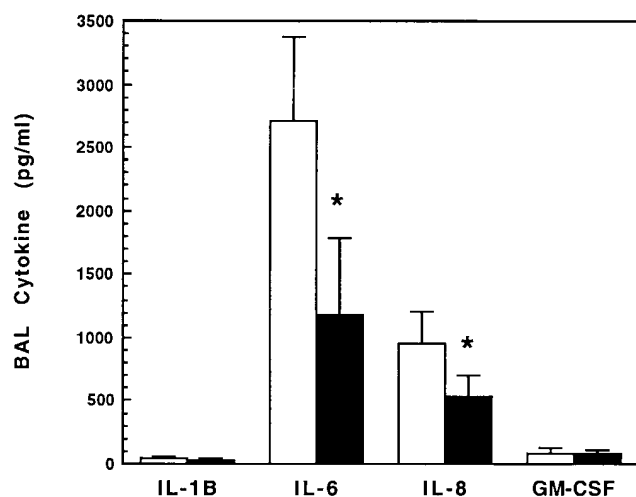
Figure 5. Mean (±SEM) levels of BAL cytokines in control (*open bars*) and CP-105,696-treated (*closed bars*) monkeys 4 h after Ag challenge on day 3 of the airway hyperresponsiveness protocol. Ag challenge caused a significant increase in BAL levels of IL-1β, IL-6, IL-8, and GM-CSF. Note: because cytokines were undetectable prior to Ag challenge, baseline levels are not presented. Treatment with CP-105,696 caused a significant reduction in BAL levels of IL-6 ($P = 0.048*$) and IL-8 ($P = 0.022*$) compared to controls.

Figure 6. Individual and mean (±SEM) MCh PC_{200} values on days 0 to 10 of the airway hyperresponsiveness study. All monkeys in both control trials developed airway hyperresponsiveness after multiple Ag challenge as indicated by the greater than threefold decrease in MCh PC_{200} before and after multiple Ag challenge. CP-105,696 prevented the significant decrease in PC_{200} MCh after multiple Ag challenge indicating that it is protective against the development of airway hyperresponsiveness. Each symbol represents a different monkey.

The only cell type that was consistently elevated on day 10 in control trials was the eosinophil (Table I). Baseline numbers of eosinophils did not significantly differ in any trial. In all three cases, eosinophils were significantly elevated on day 10, and the number of post Ag BAL eosinophils did not significantly differ in any trial. Thus, CP-105,696 did not prevent the Ag-induced increase in eosinophils. The numbers of other leukocytes that were present in the BAL fluid collected before and after multiple Ag challenge are also presented in Table I. No other cell type was significantly elevated 3 d after the last Ag challenge in any trial.

In addition to leukocyte numbers in the BAL fluid, circulating leukocytes and other hematological parameters were quantified. Baseline values did not significantly differ for any measurement. There was no significant change in total peripheral blood leukocyte (WBC), erythrocyte (RBC) or platelet (Plt) number after Ag challenge in either control or CP-
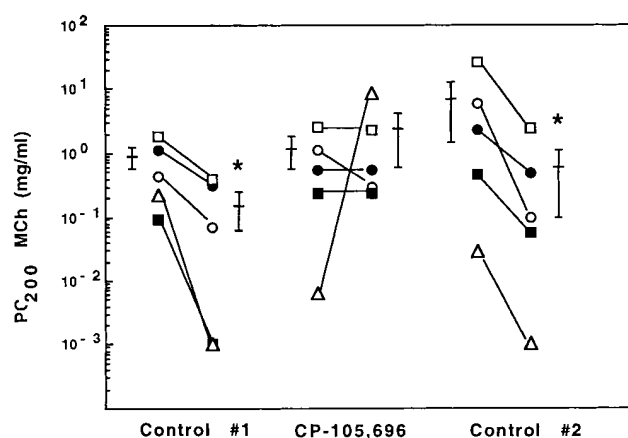
105,696 treatment trial. The same is true for both hemoglobin (Hb) and hematocrit (Hct) measurements. Pre-Ag peripheral blood eosinophils were not significantly different in any trial (control 1: $3.11\pm0.86 \times 10^3/\mu l$; CP-105,696: $2.57\pm0.91 \times 10^3/\mu l$; control 2: $3.39\pm1.06 \times 10^3/\mu l$). After Ag challenge, eosinophils were significantly increased in the peripheral blood of both controls ($5.68\pm0.93 \times 10^3/\mu l$ and $5.17\pm1.56 \times 10^3/\mu l$) and monkeys treated with CP-105,696 ($5.39\pm1.90 \times 10^3/\mu l$) indicating that CP-105,696 did not reduce the peripheral blood eosinophil response to Ag. This is consistent with observations of elevated eosinophil number in the BAL fluid.

Curiously, peripheral blood basophils were significantly reduced after multiple Ag challenge in the CP-105,696 treatment trial, but not in either control trial. We believe this to be a sampling artifact due to the relatively low number of this cell type in monkeys. There was a trend for neutrophils to be elevated and monocytes and lymphocytes to be reduced after multiple

Table I. Mean (±SEM) BAL Differential Leukocyte Counts Before (day 0) and After (day 10) Multiple Ag Challenge in Both Control Trials and in CP-105,696-treated Monkeys

Cell type	Control 1		CP-105,696		Control #2	
	Day 0	Day 10	Day 0	Day 10	Day 0	Day 10
Eos	8.52×10^3	$1.39 \times 10^{5*}$	1.26×10^4	$2.34 \times 10^{5*}$	1.94×10^4	$1.52 \times 10^{5*}$
	(6.4×10^3)	(0.65×10^5)	(0.69×10^4)	(0.82×10^5)	(0.12×10^4)	(0.60×10^5)
Neut	1.80×10^3	2.97×10^3	1.56×10^4	1.50×10^4	2.12×10^4	2.71×10^4
	(1.12×10^3)	(1.23×10^3)	(1.43×10^4)	(6.47×10^3)	(1.84×10^4)	(2.18×10^4)
Mono/Macro	1.44×10^5	1.42×10^5	2.57×10^5	2.63×10^5	1.44×10^5	1.92×10^5
	(0.57×10^5)	(0.29×10^5)	(1.66×10^5)	(0.84×10^5)	(0.34×10^5)	(0.59×10^5)
Lymph	1.29×10^3	2.47×10^3	2.44×10^3	3.82×10^3	1.05×10^3	2.19×10^3
	(0.46×10^3)	(1.33×10^3)	(0.98×10^3)	(1.08×10^3)	(0.39×10^3)	(0.88×10^3)

There was no significant difference in cell type in control and CP-105,696 trials. *Eos*, eosinophils; *Neut*, neutrophils; *Mono/Macro*, monocytes + macrophages; *Lymph*, lymphocytes. $P < 0.05$, compared with pre-Ag number on day 0.

Ag challenge in controls and treated monkeys, but these differences were not statistically significant.

Discussion

CP-105,696 inhibited neutrophil chemotaxis and the upregulation of CD11b in primate neutrophils in a manner similar to that previously demonstrated for human neutrophils (8). In vivo, treatment with CP-105,696 protected against Ag-induced neutrophil influx and increases in the BAL levels of IL-6 and IL-8. CP-105,696 treatment prevented the development of AHR after multiple Ag challenge, but did not inhibit the pulmonary infiltration of any leukocyte type, including eosinophils, during this procedure.

Previously, investigators demonstrated that when neutrophils are stimulated with LTB_4 in vitro, there is an exocytosis of certain intracellular granules (14) and one consequence of this is the upregulation of the surface adhesion molecule $\beta 2$ integrin, Mac-1 (CD11b/CD18). Our in vitro results demonstrated that CP-105,696 exerted an inhibitory effect on LTB_4-mediated CD11b upregulation and consequent neutrophil chemotaxis as predicted and at a similar potency to that observed in humans. These results were entirely consistent with our observation in vivo that CP-105,696 inhibited the increase in BAL neutrophils 4 h after a single Ag challenge. Our in vivo results further suggest that LTB_4 is a critical, though not exclusive, mediator of Ag-induced neutrophil chemotaxis since CP-105,696 reduced, but did not eliminate the Ag-induced increase in BAL neutrophils. Similar results were obtained in atopic asthmatics treated with another LTB_4 antagonist, LY-293111 (15).

Treatment with CP-105,696 also decreased levels of IL-6 and IL-8 after Ag challenge. 5LO inhibitors can reportedly reduce IL-6 production by various cell types in vitro (16, 17), and we have observed similar effects after Zileuton treatment in our in vivo model (unpublished observation). IL-6 is a proinflammatory cytokine produced by a variety of cells including monocytes. macrophages, fibroblasts, vascular endothelial cells, keratinocytes, T cells (reviewed in reference 8), mast cells (16) and epithelial cells (17). IL-6 is involved in the final differentiation of B cells into immunoglobulin secreting cells and enhances IL-4–induced IgE synthesis by B cells (reviewed in reference 18). IL-6 increases the cytotoxic activity of T cells and plays a major role in the acute phase response of the liver (reviewed in reference 19). IL-6 also has a positive hematopoietic effect in the bone marrow (16).

The reduction of IL-6 levels in the BAL fluid by may be secondary to the inhibition of LTB_4. Brach et al. (16) determined that LTB_4 induces the synthesis of IL-6 by human monocytes through activation of the IL-6 gene via the transcription factor, NF-kB and, to a lesser extent, NF-IL6. LTB_4 stimulation not only resulted in the accumulation of IL-6 transcripts, but also in the amount of IL-6 protein produced. The reduction of IL-6 by CP-105, 696 that we observed in vivo, is consistent with these in vitro results.

Treatment with CP-105,696 also reduced BAL levels of IL-8. Previously, McCain et al. (20) demonstrated that LTB_4 stimulates a dose-related production of IL-8 from human peripheral blood polymorphonuclear leukocytes. Taken together, these observations suggest that LTB_4 mediates Ag-induced increases in BAL IL-8 and the blockade of this pathway by CP-105,696 may have further contributed to the protective effects of this compound. IL-8 is a potent chemoattractant and activator of neutrophils in primates (21) and purportedly induces eosinophil chemotaxis in human asthmatics (22). Furthermore, intradermal administration of IL-8 in rabbits causes plasma exudation (23). These results are quite interesting because it has been shown that both IL-6 and IL-8 are elevated in the lavage fluid of asthmatics that develop a late phase response to antigen (24). Based on these observations, suppression of IL-6 and IL-8 may have had a protective effect against subsequent alterations in airway funtions normally observed using this protocol.

Given the observation that LTB_4 injection into rabbit skin caused microvascular leak (23), we expected that treatment with CP-105,696 would reduce protein levels in the BAL fluid since this end point purportedly reflects vascular leak. Although not statistically significant, CP-105,696 tended to reduce levels of BAL protein. It may be that other mediators released after Ag challenge also contribute to this effect and the blockade of LTB_4 receptors may not be sufficient to prevent this response. Since edema is a feature of asthmatic airways (25), the tendency for CP-105,696 to reduce protein leak may have contributed somewhat to its efficacy in our model.

The effects of CP-105,696 on the chronic responses to Ag challenge are especially interesting since these responses reflect more closely the asthmatic condition. We have already alluded to the ability of LTB_4 to induce AHR in other animal models (3, 4). Thus, we would expect that treatment with CP-105,696 would prevent Ag-induced AHR if LTB_4 was a contributing mediator to this response, which, in fact, was the case.

Gundel et al. (26) reported that neutrophils are requisite for the late phase response (LPR) to Ag in primates since blockade of neutrophil chemotaxis by a 5LO inhibitor prevented the occurrence of the LPR. Given the long-standing association between the development of the LPR and AHR observed by many investigators (27), perhaps a leukotriene-mediated component, sensitive to both LTB_4 antagonism and 5LO inhibition, underlies both the LPR and also contributes to the development of AHR. In their evaluation of the 5LO inhibitor, BI-L239, Wegner et al. (28) found the LPR was blocked in primates and sheep and AHR was reduced in sheep and guinea pigs. Neutrophil infiltration was also reduced in both sheep and primates. These effects may have been partially elicited by the reduction of LTB_4 levels and, if so, would be entirely consistent with our observations.

The current study represents the first instance in which we observed the prevention of the AHR without an accompanying reduction in BAL eosinophil number. CP-105,696 inhibited LTB_4-induced eosinophil chemotaxis into the skin of guinea pigs (8) and also reduced IL-8, a puported eosinophil chemoattractant (22). Based upon these results, one may have expected CP-105,696 treatment to reduce the increase in BAL eosinophils in the current study. However, this expectation assumes that LTB_4 is an important mediator of eosinophil chemotaxis when, in fact, other mechanisms may predominate. Several studies in which LTB_4 antagonists were evaluated in guinea pigs (29) or humans (15) reported that these compounds had no effect on Ag-induced eosinophil infiltration; our results are completely consistent with these. Given that Ag challenge elicits numerous, redundant mechanisms by which eosinophils can infiltrate the lung, it is unlikely that LTB_4 antagonism could inhibit all of these pathways.

The apparent dissociation between eosinophilia and AHR, though not expected, is not unprecedented. In guinea pigs treated with U-75,302, AHR was reduced without altering the eosinophil response or EPO levels in the BAL fluid (29). Although LTB_4 caused the development of AHR in dogs, it did not significantly elevate eosinophils at the time AHR was noted (4). Both these studies and our own suggest that the mere elevation in eosinophil number may be less meaningful than the activation state of the eosinophils themselves. It is possible that even though eosinophils are present in CP-105,696-treated monkeys, they may not be releasing the constituents that cause altered airway function. Our attempts to assess the activation state of eosinophils have been plagued by lack of cross-reactivity between human reagents and monkey eosinophils. We will continue our attempts to assess the activation state of esoinophils however, as we believe it may provide an explanation for our current observations.

On the basis of these results, we believe that LTB_4 inhibition represents a valid therapeutic approach to the treatment of asthma by virtue of its ability to prevent the development of AHR and its reduction of proinflammatory cytokines after antigen challenge. Although CP-105,696 did not reduce the Ag-induced increase in eosinophil number, we speculate that the eosinophils present were functionally compromised such that their constituents could not contribute to altered airway function.

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