The inhibitory effects of topically active glucocorticoids on IL-4, IL-5, and interferon-γ production by cultured primary CD4$^+$ T cells

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This study was conducted to directly compare the in vitro efficacy and potency of several glucocorticoids in inhibiting T-cell cytokine production. The glucocorticoids tested were fluticasone propionate, budesonide, triamcinolone acetonide, and beclomethasone dipropionate, which are currently inhaled therapies for the treatment of allergic airway disease. Also used were betamethasone phosphate and the newly developed mometasone furorate. With a novel cell culture system, purified peripheral blood CD4$^+$ T cells from normal donors were stimulated with immobilized anti-CD3 and soluble anti-CD28 monoclonal antibodies to induce high levels of IL-4, IL-5, and interferon-γ. By cell sorting, it was found that the IL-5 produced originated from memory cells, whereas both memory and naive cells produced interferon-γ. Mometasone and fluticasone inhibited IL-5 and IL-4 similarly (mometasone IL-5 inhibitory concentration of 50% = 0.27 ± 0.1 nmol/L and IL-4 = 0.19 ± 0.08 nmol/L). For both cytokines, the results indicate that mometasone and fluticasone were more potent than beclomethasone, triamcinolone, budesonide, and betamethasone. Of clinical importance is the finding that all steroids demonstrated less efficacy versus interferon-γ than IL-4 and IL-5. Glucocorticoid reduction of Th2 cytokines with lesser effects on interferon-γ would serve to reverse the exaggerated Th2 response that contributes to pathophysiology observed in allergic disease. Therefore the use of topically active glucocorticoids with low systemic bioactivity for the treatment of allergic inflammation may be particularly effective in modulating the cytokine activity that is an important component of the allergic response.
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Key words: Glucocorticoid, cytokine, IL-4, IL-5, interferon-γ, naive T cell, memory T cell

Abbreviations used

BDP:	Beclomethasone dipropionate
BETA:	Betamethasone phosphate
DMSO:	Dimethyl sulfoxide
FACS:	Fluorescence-activated cell sorter
FP:	Fluticasone propionate
GC:	Glucocorticoid
IC_{50}:	Inhibitory concentration of 50%
IC_{30}:	Inhibitory concentration of 30%
IFN-γ:	Interferon-γ
mAb:	Monoclonal antibody
MF:	Mometasone furoate
PBMCs:	Peripheral blood mononuclear cells
PBS:	Phosphate-buffered saline
TA:	Triamcinolone acetonide

Glucocorticoids (GCs) have been potent and effective drugs for the treatment of severe allergic disease since their discovery decades ago. More recently, inhaled GCs with increased potency and reduced systemic bioavailability have become the preferred treatment for milder forms of asthma.[1, 2] GCs have multiple, well-characterized means by which they ameliorate allergic airway symptoms, including alteration of cell trafficking of lymphocytes and inflammatory cells such as eosinophils and basophils, blockade of mediator release from inflammatory cells,[2, 3] and inhibition of cytokine production or activity.[4, 5]

Relatively few studies have directly compared the GCs, which are currently used for inhaled therapy of allergic airway disease, for inhibitory effects on cytokine production. This study was conducted to evaluate the in vitro efficacy and potency of several clinically relevant GCs in inhibiting T-cell production of IL-4, IL-5, and interferon (IFN)-γ with purified peripheral blood CD4$^+$ T cells from normal donors. The GCs used were fluticasone propionate (FP), budesonide, triamcinolone acetonide (TA), and beclomethasone dipropionate (BDP), all relatively new GCs used as inhaled treatments for asthma.[6] In addition, betamethasone phosphate (BETA), an older generation GC with good oral activity in multiple species, was tested.[7] Also included was mometasone furoate (MF), a highly potent GC with low oral activity, which is under development as an inhaled preparation for the treatment of inflammatory airway disorders.[8]

Many studies of allergic conditions have reported an association of inflammation with elevated expression of cytokines, in particular IL-4 and IL-5. Allergic immune responses involving elevated IgE levels, increased mast cell activation and mediator release, and selective differentiation of eosinophils are regulated by IL-4 and IL-5.[9, 10] Increased activation of CD4$^+$ T cells and

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increased T-cell expression of IL-4 and IL-5 but not IFN-γ mRNA in bronchoalveolar lavage from allergen challenge of patients with atopic asthma[11, 12] and patients with nonatopic asthma[13] has been observed. Bronchial mucosal biopsy specimens from patients with asthma, but not normal volunteers, show increased IL-5 mRNA expression and a correlation with increased numbers of mucosal eosinophils and activated T cells.[14] A preferential activation of cells expressing a Th2 pattern of cytokines (IL-3, IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor) but not Th1 cytokines (IL-2 and IFN-γ) has been seen in allergen-induced late-phase cutaneous reactions in atopic patients.[15]

Immunohistochemical and in situ hybridization studies of tissue from several allergic states have shown that cell types other than T cells synthesize IL-4 and IL-5. Both mast cells and eosinophils produce these cytokines.[16-18] The relative role of the different cell sources of Th2 cytokines in allergic disease is difficult to assess experimentally. However, in vivo depletion of T cells in a mouse model of pulmonary inflammation reduced pulmonary eosinophilia and the elevated levels of IL-4 and IL-5 mRNA associated with the inflammation, indicating a strong T-cell dependency of these responses.[19] Therefore therapies aimed at reducing T-cell–derived IL-4 and IL-5 secretion would be of benefit in reducing allergic inflammation.

In these studies GC effects on purified, cultured peripheral blood $CD4^+$ T cells from normal donors were studied. Optimal production of IL-4 and IL-5 by these T cells required stimulation with immobilized anti-CD3 monoclonal antibody (mAb) and a costimulatory signal provided by soluble anti-CD28 mAb. These stimuli mimic those provided physiologically through T-cell receptor signaling after interaction with specific antigen in the context of class II major histocompatibility complex molecules on accessory cells and through costimulatory receptor molecule (CD28, CTLA-4) signaling by interaction with counterreceptors (CD80 and CD86), also on accessory cells.[20] The contribution of two subpopulations of $CD4^+$ T cells, memory and naive, to cytokine secretion was determined by cell sorting before cell culture and stimulation for cytokine secretion. It was within this well-characterized system that the GCs were evaluated for inhibition of cytokine production.

METHODS

Lymphocyte isolation and Dynabead separation of $CD4^+$ cells

Peripheral blood was obtained from normal, healthy individuals at the Schering-Plough Research Institute. Freshly drawn, heparinized human blood was diluted 1:1 with Hanks' balanced salt solution (Fisher Scientific, Springfield, N.J.), and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. After three washes with Hanks' balanced salt solution, cells were counted and adjusted to 1×10^7 cells/ml in phosphate-buffered saline (PBS) containing 2% fetal calf serum (Gibco BRL, Gaithersburg, Md.) for subsequent isolation of $CD4^+$ T cells, or PBMCs were stimulated directly for cytokine production.

$CD4^+$ T cells were isolated from the PBMCs with Dynabeads M450 (Dynal Corp., Lake Success, N.Y.) at a 3:1 bead to cell ratio. $CD4^+$ cells were then removed from the Dynabeads by addition of a 1:10 dilution of Detachabead M450 solution (Dynal Corp.). $CD4^+$ T-cell separation and bead detachment were performed according to the manufacturer's specifications. The cells were resuspended to a final concentration of 1×10^6 cells/ml in complete RPMI-1640 medium containing 10% fetal calf serum, 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 2 mmol/L L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin (Fisher Scientific) and 5×10^{-5} mol/L 2-mercaptoethanol (Sigma, St. Louis, Mo.). Fluorescence-activated cell sorter (FACS) analysis demonstrated that $CD4^+$ T cells purified by Dynabeads were routinely more than 98% pure. $CD4^+$ T cells can be distinguished further into unprimed naive and memory cells on the basis of their relative expression of CD45RA and CD45RO.[21, 22] FACS analysis of $CD4^+$ T cells isolated as described above contained approximately 45% naive ($CD4^+CD45RA^{hi}CD45RO^{lo}$) and 40% memory ($CD4^+CD45RA^{lo}CD45RO^{hi}$) cells.

$CD4^+$ cell culture

Six-well Falcon tissue culture plates were coated with 10 μg/ml anti-CD3 mAb (clone UCHT1; Pharmingen, San Diego, Calif.) in PBS and incubated overnight at 4° C. The plates were washed with cold PBS three times just before use. The $CD4^+$ cells were added to the anti-CD3–coated plates at 4×10^5 cells/ml, 4 ml/well, and incubated for 3 days at 37° C in 5% CO_2. On day 3, the cells were removed from the plates, washed, and diluted to a final concentration of 4×10^5 cells/ml in complete RPMI-1640 medium containing recombinant human IL-2 (20 U/ml; Biosource, Camarillo, Calif.) and recultured in uncoated six-well Falcon tissue culture plates for 3 days. On day 6, the cells were resuspended in complete RPMI-1640 medium without additional IL-2 and stimulated as indicated. Over the 6-day culture period, the $CD4^+$ T cells expanded approximately 5-fold to 10-fold and remained highly pure (>98%), indicating that no contaminating cell population grew during the culture period.

Cell stimulation for cytokine production

Freshly isolated PBMCs or $CD4^+$ T cells grown for 6 days as described above were harvested, washed, and resuspended at 1×10^6/ml in complete RPMI-1640 medium. To 100 μl of cells, stimuli to induce cytokine secretion were added. Stimuli included phorbol myristate acetate (Sigma) or anti-CD3 and anti-CD28 mAbs (Pharmingen) in various combinations as indicated. For mAb stimulation, 100 μl of cells was added to washed anti-CD3–coated (10 μg/ml) microtiter plates, and anti-CD28 mAb was added to a final concentration of 1 μg/ml. For all groups a total of 200 μl was used per well. All controls and experimental groups were tested in quadruplicate. GCs were prepared in dimethyl sulfoxide (DMSO) and added to the microtiter plates at culture initiation at the indicated final concentrations. Typically, each GC was titrated with threefold dilutions over 3 to 6 logs. DMSO control groups, which contained solvent concentrations equivalent to that in the highest concentration of GCs, were included and were always equal to or less than 0.025%. The following GCs were used: MF, BETA, and FP (synthesized at Schering-Plough); BDP (Sigma); budesonide (Farmabios, Pavia, Italy); and TA (Sigma). The cultures were incubated for 48 hours at 37° C in 5%

TABLE I. Cytokine levels in $CD4^+$ T-cell cultures

	IL-5	IL-4	IFN-γ
Mean*	2376	3580	4551
SEM	531	933	975
Range	477-5414	846-11481	1978-9897
n†	9	6	8

*Mean cytokine levels (in picograms per milliliter) ± SEM in donors. Values from individual donors were determined in quadruplicate. SEM for quadruplicates for an individual donor was ≤ 15% of mean.
†Number of donors of $CD4^+$ T cells tested.

CO_2, at which time supernatants were harvested for ELISA. Primary and secondary antibodies for IL-5 and IL-4 quantitation by ELISA were obtained from Pharmingen and used according to their protocol; the detection limit for these assays was approximately 20 pg/ml. IFN-γ was measured by using an IFN-γ Cytoscreen Immunoassay Kit (Biosource); the limit of detection was 4 pg/ml according to the manufacturer's specifications.

A total of nine normal donors was used for isolation of $CD4^+$ T cells (with the Dynabeads M450 method) and measurement of GC inhibition of cytokine secretion (Tables I to III). Measurement of IL-5 was done for all nine donors, and IL-4 and IFN-γ ELISAs were performed for six and eight of the same donors, respectively. Percent inhibition was calculated for each concentration of GC for each cytokine as follows: [(DMSO solvent control − GC group) ÷ DMSO solvent control] × 100. The concentration of GC that caused 50% or 30% inhibition of the cytokine level in DMSO solvent control cultures is designated the IC_{50} or IC_{30}, respectively.

FACS staining, sorting, and analysis

Unseparated PBMCs, freshly isolated $CD4^+$ cells, or cultured $CD4^+$ T cells were prepared at a concentration of 1×10^7 cells/ml in 2% fetal calf serum–PBS (staining buffer). Fc-mediated and nonspecific binding was blocked by an initial 10-minute incubation with anti-Fc receptor antibody, 2.4G2 (PharMingen). Cells were subsequently stained at recommended concentrations for 25 minutes on ice with the indicated antibody-fluorochrome conjugates (Becton-Dickinson Immunocytometry Systems, San Jose, Calif., with the exception of anti-CD4-Tricolor from Caltag, S. San Francisco, Calif.). After washing with staining buffer twice, cells were fixed with 1% paraformaldehyde.

All cell samples were analyzed for fluorescence by using a FACSort equipped with a Cyonics air-cooled argon ion laser with standard optics (Becton-Dickinson). Dead cells were excluded by gating on the basis of forward light scatter and side angle scatter. In all experiments fluorescence was measured in linear amplification and optimized with appropriate photo multiplier tube (FL1, FL2, FL3) and compensation settings to provide the best resolution between different fluorescent signals. Data analysis was done with the CellQuest Software Analysis Package (Becton-Dickinson). Cell sorting and analysis were done on a FACS Vantage flow cytometer equipped with Coherent Enterprise and Coherent Spectrum lasers. For sorting, freshly isolated PBMCs were stained as described above, without paraformaldehyde fixation, with the following antibody-fluorochrome conjugates (Becton-Dickinson): anti-CD4-Tricolor, anti-CD45–fluorescein isothiocyanate, and anti-CD45RO phycoerythrin. After gating for live cells on the basis of forward light scatter and side angle scatter, a sort region was established to exclude all $CD4^-$ cells. $CD4^+$ T cells were collected by using this sort gate. Additional sort gates were defined, and cells were sorted simultaneously for naive ($CD4^+CD45RA^{hi}CD45RO^{lo}$) and memory ($CD4^+CD45RA^{lo}CD45RO^{hi}$) $CD4^+$ cells.[21, 22] After sorting, the cells were pelleted by centrifugation and resuspended in staining buffer; reanalysis was done on an aliquot to determine purity. Sorted total $CD4^+$, naive $CD4^+$, and memory $CD4^+$ T cells were cultured for 6 days as described above, after which time naive and memory cell phenotypes were redetermined.

TABLE II. Steroid inhibition (IC_{50}) of cytokine secretion

Steroid	IL-5*	IL-4*
MF	0.27 ± 0.1	0.27 ± 0.15
FP	0.19 ± 0.08 (NS)	0.32 ± 0.16 (NS)
BDP	8.2 ± 1.7†	11.0 ± 5.9†
TA	9.8 ± 5.1†	11.1 ± 5.8†
Budesonide	1.7 ± 0.7†	7.7 ± 6.5
BETA	35.7 ± 13†	100 ± 51†
n‡	9	6

NS, Not significantly different from MF.
*IC_{50} in nanomoles per liter (±SEM) for IL-5 and IL-4. For IFN-γ, in four of eight donors maximum inhibition equaled 50% to 80%, and four of eight donors showed less than 50% maximum inhibition. Therefore IC_{50} value could not be obtained for inhibition of IFN-γ.
†The indicated steroid is significantly less potent than MF ($p < 0.05$).
‡Number of donors used.

Statistical analysis

For both IL-4 and IL-5, IC_{50} and IC_{30} values for the different treatments were compared by using the two-sided Wilcoxon signed-rank test.[23] Significance tests were performed at the 0.05 level of significance. The *p* values presented are unadjusted for multiple comparisons.

RESULTS

The experiments performed in this study were done to evaluate the effects of several GCs, used clinically for the treatment of allergic disease,[6] on cytokine secretion by T cells. It was of interest to study the effects of these GCs on Th2 cytokines, particularly IL-4 and IL-5, because they are known to play a role in allergic inflammation,[9] as well as IFN-γ, a Th1 cytokine,[10] which can downregulate the effects of Th2 cytokines.[24] We chose to use polyclonal populations of T cells, derived from multiple donors, to represent a broad T-cell repertoire, rather than a more limited number of in vitro cultured T-cell clones. Initial studies to measure cytokine levels in the absence of GCs were done by using PBMCs stimulated with immobilized anti-CD3 mAb plus soluble anti-CD28 mAb, stimuli that mimic those delivered in vivo through the T-cell antigen and CD28 receptors after interaction of T cells with antigen-presenting cells.[20] In only one of 13 tests (1 of 11 donors) was IL-5 detected in PBMCs stimulated by immobilized anti-CD3 alone. In addition, in only seven of 13 tests was measurable IL-5 detected in anti-CD3–plus anti-CD28–stimulated PBMC cultures. In these seven donors IL-5 levels ranged from 144 to 3368 pg/ml. Although indicating that costimulation in-

TABLE III. Steroid inhibition (IC_{30}) of cytokine secretion

Steroid	IL-5*	IL-4*	IFN-γ†
MF	0.046 ± 0.027	0.044 ± 0.02	0.011 ± 0.003
FP	0.028 ± 0.017 (NS)	0.054 ± 0.03 (NS)	0.052 ± 0.049
BDP	2.63 ± 0.65‡	3.54 ± 1.71 (NS)	5.7 ± 4.7
TA	3.5 ± 1.86‡	2.9 ± 1.6‡	1.23 ± 0.61
Budesonide	0.55 ± 0.22‡	1.86 ± 1.5 (NS)	0.63 ± 0.47
BETA	13.7 ± 5.0‡	26.5 ± 15.4‡	14.4 ± 9.1
n§	9	5-6	4

NS, Not significant.
*IC_{30} values in nanomoles per liter (±SEM) were determined in the same donors used for IC_{50} values (Table II).
†Values were derived from the four of eight donors in whom maximum inhibition attained was 50% to 80%.
‡The indicated steroid is significantly less potent than MF ($p < 0.05$).
§Number of donors used.

creased IL-5 production, this method of stimulation of PBMCs did not yield a reliable system with which to profile the inhibitory effects of GCs. In contrast, all donors produced IFN-γ when stimulated by immobilized anti-CD3 mAb alone (range = 109 to 3660 pg/ml), and in all cases increased IFN-γ (range = 1128 to 10134 pg/ml) was observed in costimulated cultures (mean fold increase was 7.4 ± 6.7 vs anti-CD3 mAb alone). Similar results were found with freshly isolated, purified $CD4^+$ T cells (data not shown).

To allow for the differentiation of $CD4^+$ T cells able to produce high levels of Th2 cytokines, a 6-day in vitro culture system was used. After 3 days of growth of purified $CD4^+$ T cells on immobilized anti-CD3 mAb and an additional 3 days in medium supplemented with IL-2,[5] the $CD4^+$ T cells expanded approximately 5-fold to 10-fold and remained highly pure (>98%).

$CD4^+$ T cells can be distinguished further into naive and memory cells on the basis of their relative expression of CD45RA and CD45RO.[21, 22] Because the effects of GCs might differ in naive or memory $CD4^+$ T-cell populations, it was of interest to determine which initial population, naive or memory, was responsible for cytokine secretion after the 6-day culture period. FACS analysis of $CD4^+$ T cells (Fig. 1, *A*) demonstrated that approximately 45% or 40% were naive ($CD4^+CD45RA^{hi}CD45RO^{lo}$) or memory ($CD4^+CD45RA^{lo}CD45RO^{hi}$) cells, respectively (Fig. 1, *B*), with the remaining cells being an intermediate phenotype. PBMCs were sorted by FACS into highly pure naive and memory $CD4^+$ T cells on the basis of expression of differing levels of CD45RO and CD45RA, cultured for 6 days, and then stimulated for induction of cytokine secretion. In Fig. 1 sort gates for whole $CD4^+$ T cells (R4, 1A) and naive (R2, 1B) and memory $CD4^+$ T cells (R3, 1B) are shown. After the 6-day in vitro culture with immobilized anti-CD3 mAb, virtually all of the cultured cells (99%) acquired the expression of CD45RO, and only 1% of the cells remained naive ($CD45RA^{hi}CD45RO^{lo}$, data not shown). This indicates that the naive cells had acquired a memory phenotype in vitro by losing the high expression of CD45RA and gaining the expression of CD45RO.[22]

Sorted cells and unfractionated sorted (total) $CD4^+$ T cells were cultured for 6 days and then stimulated for cytokine production with immobilized anti-CD3 mAb in the absence or presence of soluble anti-CD28 mAb or phorbol myristate acetate. As shown in two experiments in Fig. 2, memory $CD4^+$ T cells account for virtually all of the IL-5 (Fig. 2, *A* and *B*) produced by total $CD4^+$ T cells. For both total $CD4^+$ T cells and sorted memory $CD4^+$ T cells, anti-CD3 plus anti-CD28 mAb stimulation was the most potent IL-5 inducer. Cultures initiated with sorted naive $CD4^+$ T cells did not secrete significant IL-5, despite the acquisition of the memory cell phenotype. In contrast, IFN-γ could be detected in cultures initiated with either naive or memory $CD4^+$ T cells (Fig. 2, *C* and *D*). In both populations the different stimuli induced IFN-γ with the same rank order of potency (anti-CD3 + anti-CD28 mAb > anti-CD3 mAb + phorbol myristate acetate > anti-CD3 mAb alone) as for total $CD4^+$ T cells. This rank order of potency in inducing IFN-γ was similar to that seen with IL-5 production from memory $CD4^+$ T cells. Interestingly, similar findings were observed for both donors, although the donors differed as to the ratio of IL-5 to IFN-γ secreted. Donor 1 (Fig. 2, *A* and *C*) secreted more IL-5 than IFN-γ (2:1) for a given stimulus, whereas the opposite ratio was seen (1:15) with donor 2 (Fig. 2, *B* and *D*). Although IL-4 was not measured in these experiments, its secretion by naive and memory $CD4^+$ T cells is known to parallel that of IL-5.[22, 25, 26]

Having defined the contribution of naive or memory $CD4^+$ T cells to IL-5 and IFN-γ secretion by stimulated cultured $CD4^+$ T cells, this culture system was used to study the inhibitory properties of GCs on cytokine secretion from total $CD4^+$ T cells from normal donors. The cells were stimulated with anti-CD3 mAb plus anti-CD28 mAb, because this stimulus induced the highest cytokine levels in total, naive, and memory $CD4^+$ T cells (Fig. 2) and mimicked the two signals important for in vivo T-cell activation.[20] A summary of the absolute amounts of IL-4, IL-5, and IFN-γ secreted from the donor $CD4^+$ T cells used in the GC experiments is shown in Table I.

For each donor tested, each GC was titrated with threefold dilutions over a 3 to 6 log concentration range to achieve maximal inhibition and to determine IC_{30} and

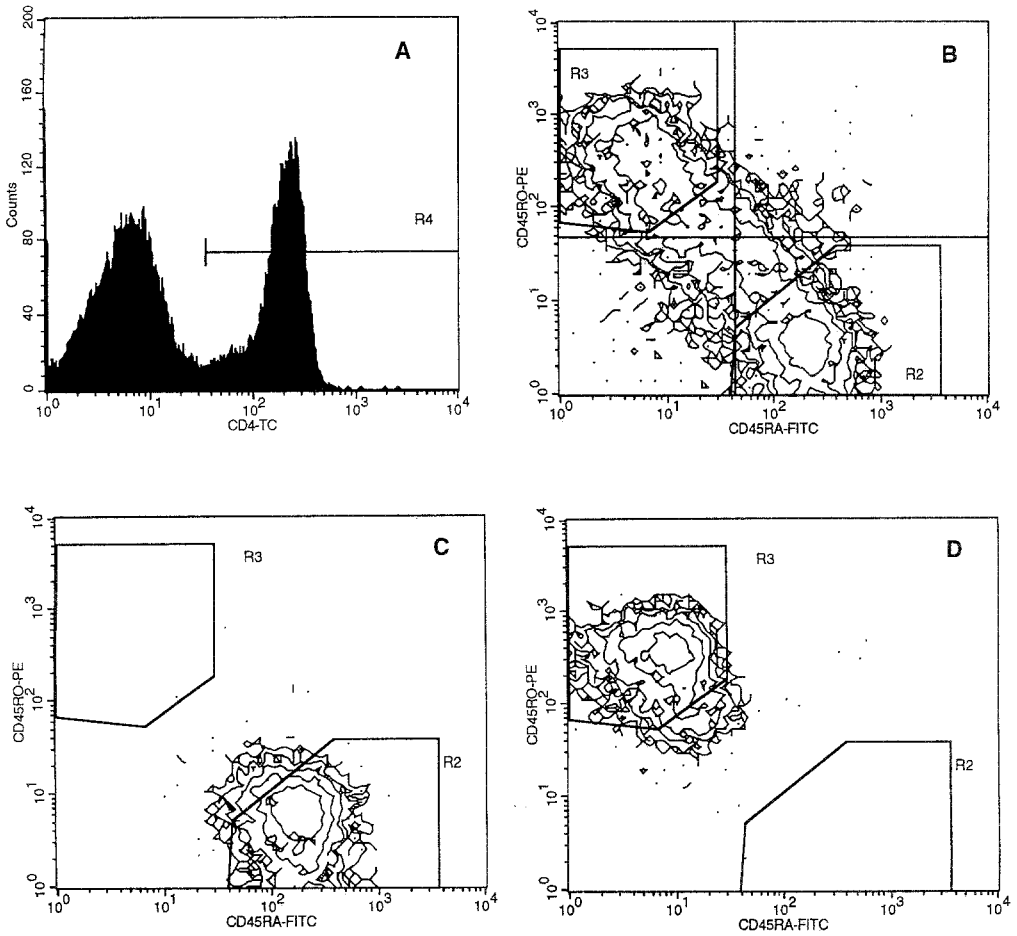

FIG. 1. Analysis of frequency of memory and naive $CD4^+$ T cells and cell sorting parameters for isolation of total $CD4^+$, naive $CD4^+$, and memory $CD4^+$ T cells. **A,** Single-parameter histogram of CD4 expression on anti-CD4-Tricolor *(TC)*-stained PBMCs, illustrating a defined peak for $CD4^+$ cells. Sort region *(R4)* was used to exclude all $CD4^-$ cells from further analysis. **B,** Two-parameter contour plot showing analysis of $CD4^+$ cells for the expression of CD45RO-phycoerythrin and CD45RA-fluorescein isothiocyanate. Sort gates *R2* and *R3* were defined, and cells were sorted simultaneously for naive ($CD4^+CD45RA^{hi}CD45RO^{lo}$) and memory ($CD4^+CD45RA^{lo}CD45RO^{hi}$) cells, respectively. The population of $CD4^+$ T cells contained approximately 45% naive cells ($CD4^+CD45RA^{hi}CD45RO^{lo}$, *lower right quadrant*) and 40% memory cells ($CD4^+CD45RA^{lo}CD45RO^{hi}$, *upper left quadrant*). **C and D,** Contour plots showing reanalysis of sorted cells. Cells defined by sort gates *R2* and *R3* appeared within their respective gate on reanalysis and were mutually exclusive. Sorted cells were greater than 98% pure. *FITC,* Fluorescein isothiocyanate.

IC_{50} values. The maximum inhibition of IL-5, IL-4, and IFN-γ achieved by the GCs varied, and this variation depended primarily on the donor. The dose-response curves for one donor are shown in Fig. 3. With the exception of one of nine donors, the maximum inhibition of IL-5 attained, regardless of the GC, was 80% or greater (Fig. 3, *A*). In contrast, GC inhibition of IFN-γ showed different patterns depending on the donor. Half of the donors (4 of 8 donors) tested showed the pattern illustrated in Fig. 3, *B*, in which 60% or greater inhibition was observed for all GCs. Three of the eight donors examined achieved less than 50% inhibition at the highest GC concentration used. Lastly, one of the eight donors examined displayed a mixed phenotype in that IFN-γ levels were resistant to inhibition by MF, FP, and BETA (maximum inhibition observed ≤40%), whereas 80% inhibition of IFN-γ levels was seen with BDP, budesonide, and TA. GC inhibition of IL-4 paralleled that of IL-5. The majority (five of six) of donors tested showed maximal inhibition of 80% or greater for all the GCs as shown in Fig. 3, *C*. GC inhibition of IL-4 from one donor displayed a mixed response; and higher inhibition (80%) was seen with BDP, TA, and budesonide than with MF, FP, or BETA (40%). This heterogeneous pattern of inhibition of different GCs on IL-4 and IFN-γ, each seen with only one of eight to nine donors, was not observed in the same donor.

Table II summarizes the IC_{50} values for all GCs. In general, maximum inhibition of IL-5 and IL-4 for all of the GCs was approximately 80% to 100% or 70% to 100%, respectively, as discussed above. The IC_{50} of MF for IL-5 was 0.27 ± 0.1 nmol/L, which was similar to that

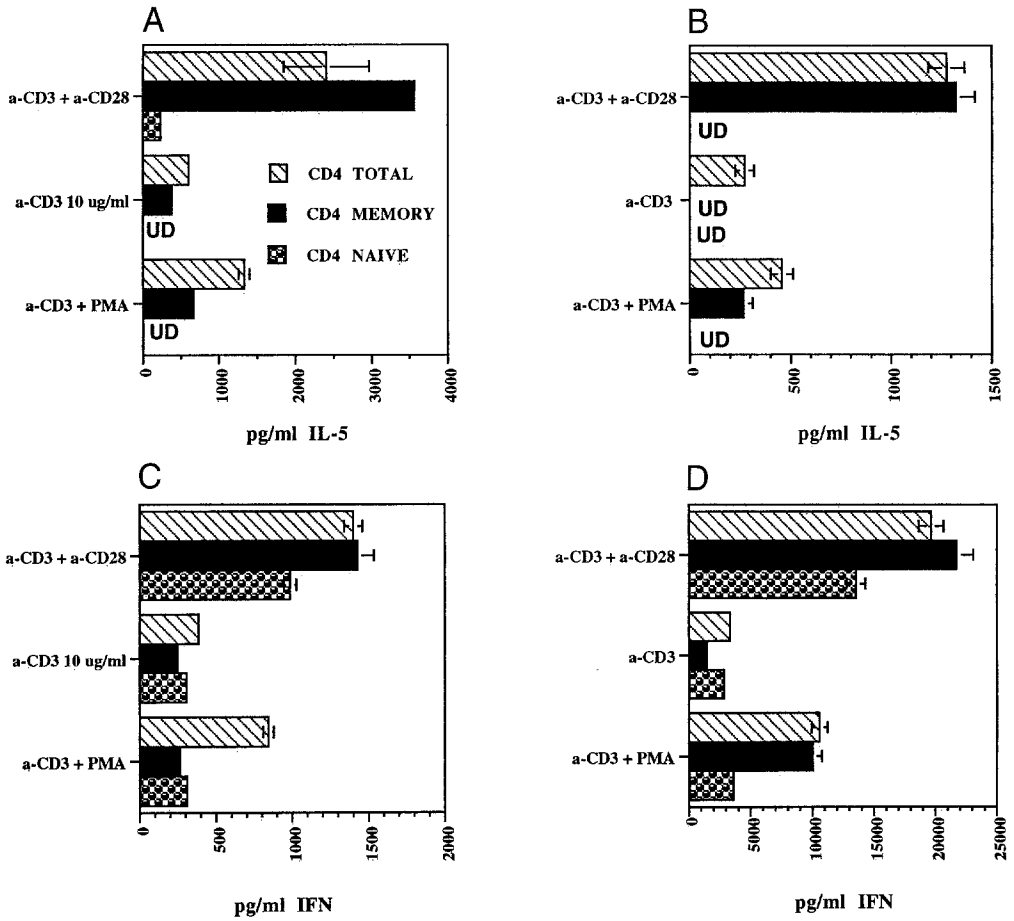

FIG. 2. IL-5 and IFN-γ secretion by cultured naive and memory $CD4^+$ T cells. Sorted total $CD4^+$, naive $CD4^+$, and memory $CD4^+$ T cells were cultured for 6 days and stimulated for cytokine production as described in the Methods. Two experiments representing two different donors (**A, C** = donor 1 and **B, D** = donor 2) are shown. Immobilized anti-CD3 and soluble anti-CD28 were used at 10 and 1 μg/ml, respectively. Phorbol myristate acetate was used at a final concentration of 1 ng/ml. Sort parameters shown in Fig. 1 were derived from donor 2. Values shown represent mean ± SEM of triplicate cultures. FACS analysis after 6-day culturing indicated that the cells were 96.8% $CD4^+$. A third experiment provided similar results. *PMA,* Phorbol myristate acetate; *UD,* below the limits of detection.

of FP (IC_{50} = 0.19 ± 0.08 nmol/L) and significantly less than that of BDP, TA, budesonide, and BETA (p = 0.0039). MF inhibited IL-4 with an IC_{50} (0.27 ± 0.15 nmol/L) similar to that of IL-5; and this was significantly less than BDP, TA, and BETA (p = 0.0312). For both cytokines MF and FP were equipotent. For IL-5 and IL-4 the rank order of potency was MF = FP > budesonide > BDP ≥ TA > BETA. There was no evidence of significant differences between the mean IL-4 and IL-5 IC_{50} values for any of the treatments.

As discussed above, the GC efficacy on IFN-γ production varied between donors. Four of the eight donors displayed 50% to 80% maximum inhibition, whereas the other four did not attain 50% inhibition. Correlations were not observed between the absolute amount of IFN-γ secreted and the maximum inhibition observed for each donor. Because of this low and inconsistent maximum inhibition of IFN-γ achieved with all GCs, IC_{50} values could not be accurately determined for this cytokine. Therefore to compare the efficacy of the GC against the three measured cytokines, IC_{30} values (in nanomoles per liter) were derived and are shown in Table III. For IFN-γ the rank order of potency, for the four donors included in the analysis, was MF > FP > budesonide > TA > BDP > BETA. Although statistical comparison could not be done because of the low number of donors, it appeared that a given GC was equally active against IL-5, IL-4, and IFN-γ. For IL-5 and IL-4, comparison of IC_{30} values generated the identical rank order of potency as for the IC_{50} values.

DISCUSSION

GCs are effective drugs for the treatment of the inflammatory component of allergic responses.[2,3] They are known to reduce the pulmonary eosinophilia associated with asthma[27] and to modulate the cytokine and T-cell involvement in asthma[27,28] and seasonal allergic rhinitis.[4] This study was performed to directly compare

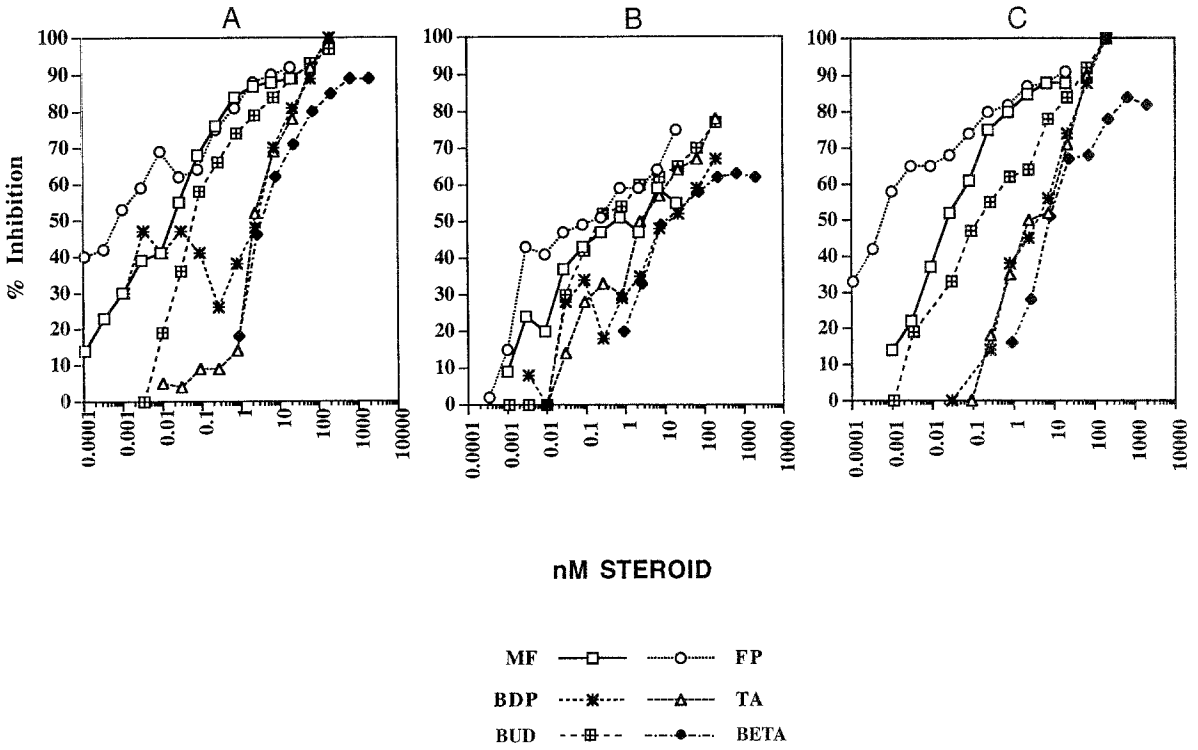

FIG. 3. GC inhibition of IL-5 **(A)**, IFN-γ **(B)**, and IL-4 **(C)** secretion by $CD4^+$ T cells from donor 1. Purified $CD4^+$ T cells were cultured for 6 days as described in Methods. Cells were stimulated with immobilized anti-CD3 plus soluble anti-CD28 mAb in the presence or absence of the indicated GC for 48 hours, at which time culture supernatants were collected. Results are shown as percent inhibition (see Methods) compared with DMSO control cultures versus GC (in nanomoles per liter) tested. Control cytokine levels (mean ± SEM pg/ml, in quadruplicate cultures) in immobilized anti-CD3 and soluble anti-CD28–stimulated cultures for this donor were: IL-5, 4046 ± 205; IL-4, 2650 ± 131; and IFN-γ, 9897 ± 594. *BUD,* Budesonide.

the effects on T-cell cytokine production of several GCs, which are marketed or in development for inhalation treatment of allergic airway disease.[3, 6]

The cell culture system used has several advantageous attributes for the accurate profiling of the inhibitory effects of GCs. First, maximal levels of IL-4, IL-5, and IFN-γ were obtained by using stimuli (anti-CD3 and anti-CD28 mAbs) that mimic physiologic signals. This stimulus has not previously been used to examine the effects of GCs on T-cell cytokine secretion. Second, the high levels of secreted cytokines allowed for the accurate determination of GC dose-response curves over a 4-log range of concentrations. Third, the contribution of different subpopulations of $CD4^+$ T cells, namely the naive and memory subsets, to cytokine production was delineated.

CD28 receptor ligation on T cells has been found to play a significant role in lung T-cell production of Th2 cytokines. In two murine models of allergic pulmonary inflammation, in vivo administration of CTLA4-Ig,[29] a soluble fusion protein that blocks CD28 interaction with its coreceptors, CD80 and CD86, on antigen-presenting cells or an antibody to CD86,[30] blocked Th2 cytokine production induced in the lungs by local allergen administration. Thus our inclusion of anti-CD28 mAb as an in vitro T-cell costimulatory signal in the GC-treated cultures used here, although not encompassing all costimulatory signals provided by accessory cells in vivo, serves to model some of the important costimulatory signals provided in vivo.

These studies demonstrate that MF and FP are highly active GCs against the Th2 cytokines IL-4 and IL-5, with in vitro IC_{50} concentrations less than 1 nmol/L. MF was equipotent with FP and in general significantly more potent than budesonide, BDP, TA, and BETA. Budesonide, BDP, and TA were also highly active preparations with nanomolar activity; whereas BETA, an older generation GC, was significantly less active in blocking cytokine production. In vivo metabolism of these GCs may alter their rank order of potency.

GCs also inhibited the production of IFN-γ, a Th1 cytokine.[10] However, because of the heterogenous donor response with large differences in maximum inhibition, the results as reported overestimate the effects of all GCs versus $CD4^+$ T-cell secretion of IFN-γ. Half of the donors tested were not included in the IC_{30} determination because only weak inhibition of IFN-γ was observed regardless of the GC, and valid statistical comparisons could not be made. Others have reported that IFN-γ, but not IL-4 or IL-5, is resistant to the inhibitory effects

of BDP[5] by using memory $CD4^+$ T cells, isolated from normal individuals and grown under conditions similar to those described here. Similar results with respect to differential inhibition of IL-4, IL-5, and IFN-γ by dexamethasone have been described in $CD4^+$ T-cell clones derived from bronchoalveolar lavage fluid or blood of normal subjects or patients with asthma.[31] Given these observations, the heterogenous donor response observed here with respect to IFN-γ inhibition by GCs may be due to donor-dependent variation[32] in the frequency of naive and memory $CD4^+$ T-cell subpopulations, each capable of producing IFN-γ and each with a different susceptibility to GC inhibition. Varying frequencies of these subpopulations across donors would have little impact on GC inhibition of IL-5 (and IL-4) because only one subpopulation, the memory $CD4^+$ T cell, produces these cytokines.

Although little is known about the sensitivity of cytokines produced by naive and memory $CD4^+$ T cells to GCs, naive $CD4^+$ T cells are more susceptible than memory $CD4^+$ T cells to the antiproliferative effects of dexamethasone, and for each subset the degree of GC-induced inhibition of proliferation was dependent on the mode of stimulation.[33] In addition, these T-cell subsets have different signaling requirements. The naive subpopulation has a higher threshold for activation provided by both the T-cell receptor and CD28 coreceptor,[34] which might be due to differences in levels of basal and induced second messenger signals.[32] Higher protein kinase C levels leading to higher levels of the GC-sensitive transcription factor AP-1, in memory as opposed to naive $CD4^+$ T cells, have been suggested to contribute to the increased GC resistance of this subset.[33]

The mechanism for this differential efficacy of GCs against various cytokines or cell subpopulations is unknown but could be due to differences in numbers of GC receptors or affinities in different T-cell subsets. Both of these parameters have been reported to contribute to an altered cytokine response pattern in patients with steroid-resistant asthma versus those with steroid-sensitive asthma.[35, 36] Importantly, in the culture system described here with polyclonal T-cell populations, it is not known, at the single-cell level, whether the Th2-type cytokines, IL-4 and IL-5, and a Th1 cytokine, IFN-γ, are coordinately produced.

This in vitro study was conducted with T-cell populations from normal, healthy volunteers. It is important that similar studies be conducted with T cells from diseased populations (i.e., asthmatic or allergic individuals) because cytokine production, GC receptor expression, affinity- or GC-mediated gene activation/repression may be dysregulated in such individuals. However, others[31] have evaluated the effects of dexamethsone on IL-4, IL-5, and IFN-γ production by bronchoalveolar lavage–and blood-derived $CD4^+$ T-cell clones from normal volunteers and patients with atopic asthma and found that IL-4 and IL-5 were inhibited to a greater extent than IFN-γ in both populations.

It is possible that the beneficial effects, with few liabilities, of inhaled GCs[37] in the treatment of allergic or asthmatic disease are due in part to reduction of IL-4 and IL-5 but not IFN-γ cytokine production. GC reduction of Th2 cytokines with lesser effects on IFN-γ would serve to reverse the exaggerated allergic response that contributes to pathophysiology. Prednisolone treatment of patients with GC-sensitive asthma decreases bronchoalveolar lavage cells expressing IL-4 and IL-5 with a concomitant increase in IFN-γ–expressing cells,[36] providing in vivo support for this concept. Therefore the results presented here indicate that the use of highly potent, topically active GCs with low systemic bioactivity, such as MF and FP, for the treatment of allergic inflammation may be particularly effective in modulating the cytokine activity that is an important component of the allergic response.

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