

Effect of prolonged use of inhaled steroids on the cellular immunity of children with asthma

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Background: Systemic corticosteroids may affect the cellular immunity, but there is no available controlled data on such effects associated with a prolonged use of inhaled corticosteroids.

Objective: The investigation was designed to study the effect of long-term inhaled beclomethasone dipropionate in daily doses of up to 600 µg on cellular immune functions.

Methods: Twenty-four children with asthma treated with inhaled beclomethasone dipropionate for a mean of 22.6 months were compared with 16 children with asthma not treated with an inhaled steroid and with 20 healthy adults. Cellular immune parameters included differential white blood count, T- and B-cell numbers, T helper and suppressor counts, T-cell mitogenic transformation, and interleukin-1 and interleukin-2 secretion.

Results: There was no difference in any of the studied cellular immune functions among the three study groups.

Conclusion: Long-term use of inhaled beclomethasone dipropionate by children with asthma, at daily doses of up to 600 µg, has no effect on certain parameters of cellular immunity. (J ALLERGY CLIN IMMUNOL 1995;95:806-12.)

Key words: Corticosteroids, beclomethasone dipropionate, asthma, cellular immunity

Recent research has made it increasingly clear that asthma is an inflammatory disease that should be treated with antiinflammatory therapy. Inhaled corticosteroids, taken daily, are now widely used as a long-term antiinflammatory regimen in treating children with asthma.¹ Although inhaled corticosteroids are considered to be safe, recent studies have demonstrated obvious systemic effects on linear growth,² on bone metabolism,^{3,4} and on the hypothalamus-pituitary-adrenal axis.^{5,6} Systemic glucocorticoid therapy may affect cellular immunity by inhibition of the synthesis and secretion of cytokines and other mediators; by depletion of

Abbreviations used

BDP:	Beclomethasone dipropionate
Con A:	Concanavalin A
CTL:	Cytotoxic T-lymphocyte line
EDF:	Euclidean Distance Function Statistics
HPAA:	Hypothalamic pituitary adrenal axis
IL:	Interleukin
MRPP:	Multiple response permutation procedures
PHA:	Phytohemagglutinin
PWM:	Pokeweed mitogen
SI:	Stimulation index
WBC:	White blood cell

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blood lymphocytes, basophils, and eosinophils; and by attenuation of T lymphocytes' reaction to various mitogens.⁷⁻⁹ This corticosteroid-induced cellular immunosuppression may result in an increased susceptibility to infections, usually of opportunistic nature (e.g., herpes zoster, *Pneumocystis carinii*, cytomegalovirus).^{10, 11}

Although as yet there is no data showing an effect of inhaled corticosteroids on the cellular

TABLE I. Patient data

	With BDP	Without BDP	Nonasthmatic Subjects
No. of Patients	24	16	20
Male/Female	21/3	10/6	12/8
Mean age (yr)	8.8	9.4	24
Range (yr)	3-18.5	2.4-17	19-35

immunity, the U.S. Food and Drug Administration has recently issued a new warning, stating that corticosteroids given by any route of administration have the potential for systemic absorption and associated immunosuppressant effects.¹² Because inhaled corticosteroids are now widely used, the potential for immunosuppression should be considered. We thus studied the effect of long-term use of inhaled beclomethasone dipropionate (BDP) on certain functions of cellular immunity.

METHODS

Patients

Parameters of cellular immunity were studied in 40 asthmatic children from the Pediatric Pulmonary Clinics of Soroka Medical Center in Beer Sheva.

A group of 24 children (21 boys and 3 girls) with asthma, aged 3 to 18.5 years (mean age, 8.8 years) constituted the BDP group. Twenty-two of them were atopic as determined by positive skin test results. They were treated twice daily with inhaled BDP (Becotide-Glaxo) 200 to 600 µg/day (mean, 382 µg/day or 16.5 µg/kg/day) for a mean of 22.6 months (range, 1 to 70 months) in addition to their usual nonsteroidal antiasthmatic drugs. All children inhaled BDP from a conventional metered-dose inhaler through a large volume spacer (Volumatic; Glaxo, U.K.) The patients in our clinic are trained at each and every visit in the proper use of their inhalers, with or without a spacer, including mouth rinsing and spitting after the treatment. Their compliance is closely monitored with diary cards and written individual treatment instructions. The control group consisted of 16 children (10 boys and 6 girls) with asthma (15 of them were atopic), aged 2.4 to 17 years (mean age, 9.4 years). They were treated with nonsteroidal antiasthmatic drugs (salbutamol and/or theophylline). Patient data are shown in Table I and individual BDP doses in Table II. An additional control group consisted of 20 healthy, untreated adults.

The protocol of this study was approved by the ethics committee of the Soroka Medical Center. The parents of each patient in the study signed a written informed consent agreement before the study began.

Protocol

Blood samples were obtained at 8:00 am, before the morning dose of BDP or other medication (12 hours

TABLE II. BDP treatment

Mean BDP daily dose (range)	382 µg (200-600 µg)
Mean BDP daily dose per kg	16.5 µg
Duration of treatment mean (range)	22.6 mo (1-70 mo)
No. of patients receiving BDP 18 mo and longer	12
No. of patients receiving BDP 36 mo and longer	8

after the previous dose). Eleven milliliters of venous blood was drawn from each patient and the following parameters were determined: differential white blood cell (WBC) count, with a Coulter automated counter (Coulter Corp., Miami, Fla.) and T-cell number, assessed by sheep red blood cell rosettes.^{13, 14} Mononuclear cells were separated on Ficoll gradient (Pharmacia, Uppsala, Sweden) and sheep red blood cell rosettes were assessed after the incubation of the mononuclear cells (10^6 in 100 µl of saline solution) with sheep red blood cells (100 µl of 1% cells in saline solution, and 50 µl of fetal calf serum preabsorbed with sheep red blood cells) for 15 minutes at room temperature and 3 hours at 4° C. The percentage of rosetting lymphocytes was determined by light microscopy.

B-cell number was assessed by the identification of membrane immunoglobulin molecules with fluoresceinated F(ab)₂ fraction of rabbit anti-human immunoglobulin antibody (OSEA 04/05; Behring, Marburg, Germany).¹³ The mixture was incubated with the fluoresceinated antibody for 30 minutes on ice, washed, and counted with the use of a fluorescent microscope.

The numbers of T-helper and T-suppressor cells were estimated by using fluoresceinated monoclonal antibodies.¹⁴ Mononuclear cells were incubated on ice for 30 minutes with Leu-3 anti-helper and Leu-2 anti-suppressor T-cell antibodies (Becton Dickinson, Mountain View, Calif.), respectively. Rabbit anti-mouse immunoglobulin F-261 (Dako, Dakopatts, Denmark) was added to the washed cells for an additional 30 minutes on ice. The cells were counted in fluorescent microscope.

T-cell mitogen transformation was studied after the incubation of mononuclear cells for 72 hours with the mitogens: phytohemagglutinin (PHA) (HA15; Wellcome, Dartford, U.K.) at a final concentration of 1:400, concanavalin A (Con A) (Miles Yeda, Rehovot, Israel) at a final concentration of 5 µg/ml and pokeweed mitogen (PWM) (Gibco, Grand Island, N.Y.) at a final concentration of 1:200 in microtiter tissue-culture plates. The cells were labeled with tritiated thymidine (TNC 29B Nuclear Research Center NEGEV) for 18 hours and harvested onto filter paper disks. The disks were counted in a beta-counter, and thymidine incorporation was expressed as counts per minute.

Secretion of interleukin(IL)-1 was assessed in a bio-

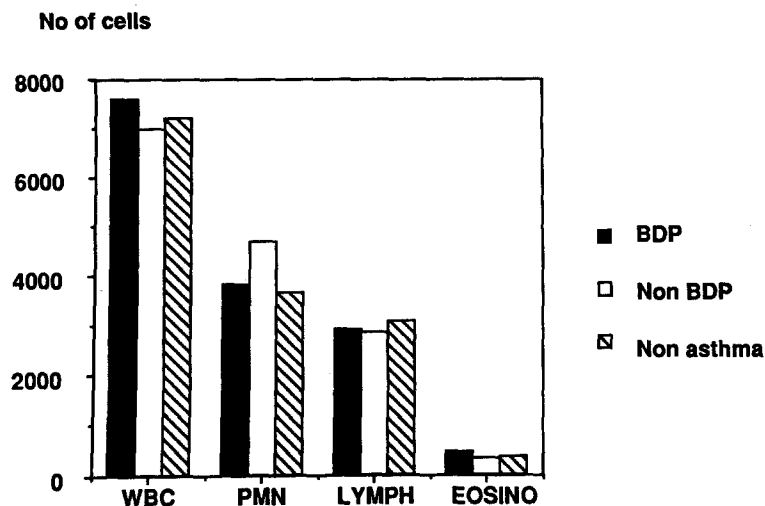


FIG. 1. Peripheral blood differential count in asthmatic patients with and without BDP therapy ($p = 0.83$, via MRPP). PMN, Polymorphonuclear neutrophils.

assay with T-cell lymphoma cells. Mononuclear cells were incubated for 24 hours with fetal calf serum containing medium with and without lipopolysaccharide. IL-1-containing supernatants from stimulated and resting cells, respectively, were incubated overnight with 1A5 cells (mouse T-cell lymphoma) and PHA. The supernatants were then incubated overnight with IL-2-dependent cytotoxic T-lymphocyte line (CTL) and pulsed with tritiated thymidine for 18 hours. Thymidine incorporation was assessed as described above.

Secretion of IL-2 was assessed in a bioassay with IL-2-dependent CTL. Mononuclear cells were incubated for 24 hours with PHA, and the IL-2-containing supernatant was then incubated with IL-2-dependent CTL cells. The IL-2 level was determined as described above.

Statistical analysis

These data have been analyzed according to a relatively new permutation technique termed multiple-response-permutation procedures (MRPP).¹⁵⁻¹⁸ MRPP is one example of the set of Euclidean distance function (EDF) statistical techniques. "Classical" statistical techniques are not appropriate for the data considered in this study; the data sets, in many of the immunologic parameters considered, do not even remotely satisfy the normal assumptions necessary for the application of such classical methods. Specifically, the data sets are generally skewed and require an artificially conceived transformation of the raw data to approximate a normal distribution (on which classical methods are based), a worrisome feature to many clinicians. In addition, the data sets are not homoscedastic as classical methods require. On the other hand, EDF techniques are non-parametric methods based on the randomization distribution approach of R. A. Fisher.^{18a} Furthermore, the EDF techniques retain the natural dimensions of the

primary measurements without any distortion caused by artificial transformations or difficult interpretations, which are foreign to many if not most biologists. For each of the parameters considered in this study, the value determined for each patient is the coordinate (on a linear scale) that identifies that patient. The 60 values, one per patient, obtained for each parameter were divided into three groups, of sizes 16, 24, and 20 patients, reflecting the group sample sizes of (1) BDP-treated children; or (2) non-BDP-treated children; or (3) normal adults, respectively. The MRPP test statistic used here arises from the within-group average distance of all pairs of points within each of the three groups. Under the null hypothesis that the three groups of 16, 24, and 20 patients could have occurred with equal chance if the 60 patients in question were allocated without replacement, the MRPP statistic provides the probability of obtaining "greater disagreement among the three groups of patients" than was observed in the actual sample. Thus the p value has the same meaning as in any test of hypothesis.

RESULTS

Sixty individuals (40 children with asthma and 20 healthy control subjects) were included in the study. Asthmatic children treated with BDP were compared with asthmatic children not treated with BDP. Fig. 1 shows that there were no significant differences in the differential WBC count regarding lymphocytes, neutrophils, and eosinophils ($p = 0.83$). Mean total WBC count was 7595 in the BDP-treated group and 6972 in the non-BDP-treated group (7200 in normal control subjects). Polymorphonuclear neutrophil counts were 3799 and 4680, respectively (3650 in the control group),

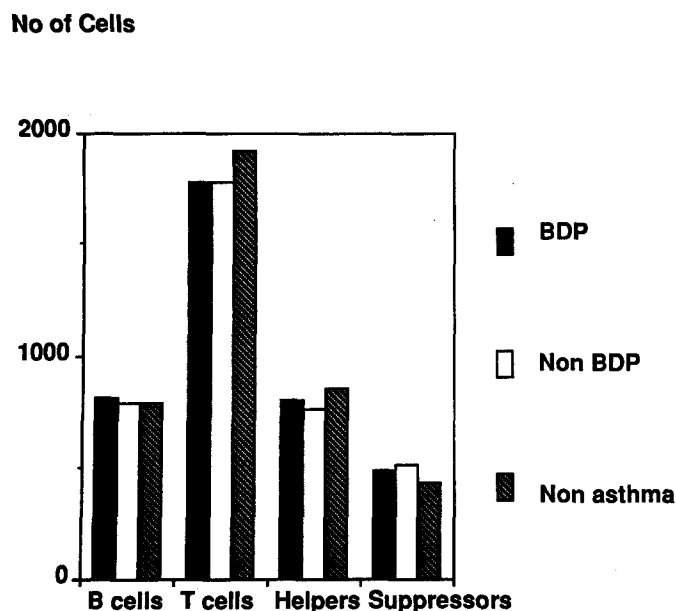


FIG. 2. Distribution of B and T cells and of subpopulations of T lymphocytes in asthmatic patients with and without BDP therapy and in control subjects ($p = 0.26$, via MRPP).

lymphocyte counts were 2914 and 2824 (3060 in the control group), and eosinophil counts were 462 and 338 in both groups (355 in the control group). Comparison of T-, B-, T-helper, and T-suppressor cell numbers again did not reveal any significant differences among the groups ($p = 0.26$) (Fig. 2), and the results were within the normal range for our laboratory. Mean B-cell count was 815 in the BDP group, 790 in the non-BDP-treated group, and 805 in the control group. T-cell counts were 1777, 1779, and 1922, respectively; T-helper cell counts were 799, 764, and 887, respectively; and T-suppressor cell counts were 479, 515, and 425, respectively. The polyclonal lymphocyte activation by mitogens (PHA, Con A, and PWM), expressed in counts per minute, is presented in Fig. 3. The comparison of four parameters was performed for each mitogen in the three groups of individuals: Δ , $\Delta\%$ (when Δ represents cpm of test – cpm of blanc and $\Delta\%$ represents Δ of the patient compared with Δ of a normal control subject studied on the same day); stimulation index (SI) (defined as cpm of test/cpm of blanc) and SI% (this value represents the SI value of the patient compared with the SI value of a normal control subject studied on the same day). The comparison did not reveal any statistically significant differences when BDP-treated and untreated groups were compared ($p = 0.127$) and when all three groups were compared ($p = 0.448$). Fig. 3 shows only the Δ cpm values for each mitogen in the three groups of patients. PHA

counts were 27,253 cpm in the BDP group, 37,120 cpm in the asthmatic non-BDP group, and 28,563 cpm in the healthy control group. PHA SI values were 125, 134, and 110, respectively. Con A counts were 8765, 11,241, and 10,843 cpm, respectively; and Con A SI values were 23.7, 57, and 42 in the three groups. PWM counts were 8065, 10,238, and 10,626 cpm; and PWM SI values were 29, 66, and 38, respectively. IL-1 secretion from stimulated or nonstimulated cells and IL-2 secretion are shown in Fig. 4. No significant difference was observed among the three groups ($p = 0.182$). Resting IL-1 values were 102,928 in the BDP group, 98,840 in the non-BDP group, and 96,548 in the healthy control group. Stimulated IL-1 values were 101,536, 102,112, and 99,583, respectively. IL-2 values were 7181, 9141, and 17,126, respectively. There was no difference in any of the parameters studied between male and female subjects; thus, the different male-to-female ratios in the different groups could not affect the results. Two of the patients have used BDP for a period of less than 3 months (one was treated for 3 months and one for 1 month). Results of their immunologic studies were not different from those of patients treated for longer periods.

DISCUSSION

Chronic inflammation appears to be a major component in the pathogenesis of asthma. The inflammation of the respiratory epithelium results

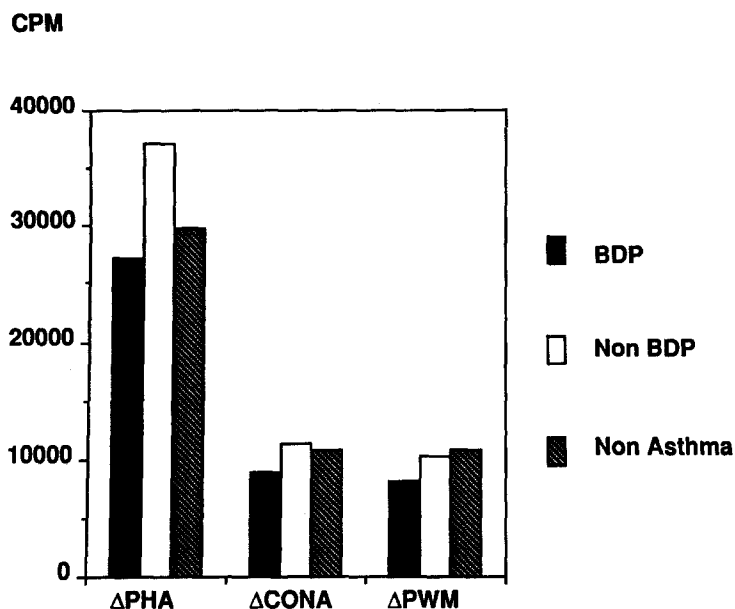


FIG. 3. Mitogen-induced mononuclear blastogenesis in asthmatic patients with and without BDP therapy and in normal control subjects ($p = 0.127$ when the two groups of asthmatic patients were compared and $p = 0.448$ when all three groups were compared). All of the statistical analyses were performed with MRPP.

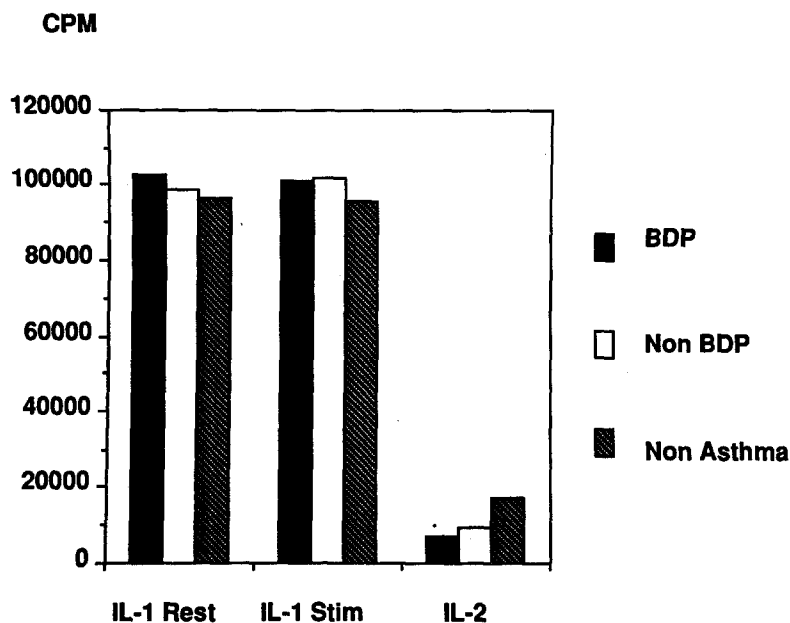


FIG. 4. Cytokine secretion from mononuclear cells of asthmatic patients with and without BDP therapy. IL-1 release was measured in resting and stimulated cells ($p = 0.182$, via MRPP).

in swelling of mucous membranes, increased secretion of viscous mucus, and enhanced plugging of the medium- and small-sized airways.¹⁹ The corticosteroids, which exhibit strong antiinflammatory effects, can reduce these processes and thus be of great use for patients with asthma. However, in

addition to the antiinflammatory effect, systemically administered corticosteroids have been shown to induce immunosuppressive effects.^{7,8,11} These effects include a rise in peripheral WBC and neutrophil counts; a decrease in lymphocytes, eosinophils, and monocytes; suppression of mito-

genic lymphocyte transformation; suppressive effect on T-cell number; T-cell sub-populations (helpers and suppressors); and prevention of lymphokine secretion. To separate the locally desired antiinflammatory effect from the systemic, undesired immunosuppressive effect, a topically inhaled steroid preparation has been developed. Inhaled steroids, used in conventional doses, have shown significant topical antiinflammatory potency and are considered to be safe with minimal systemic effects.²⁰ The use of inhaled steroids has not increased the incidence, duration, or severity of viral fungal or bacterial respiratory infections in patients with uncomplicated asthma.²¹ Recently, there have been anecdotal reports about a coincidence of tuberculosis²² with use of inhaled beclomethasone and of severe varicella infection with intranasal administration of this drug.²³ As a consequence, the Food and Drug Administration has requested that all manufacturers of oral, injectable, and inhaled corticosteroids make labeling changes to warn physicians about a potential hazard in treatment with corticosteroids in patients who have varicella and other viral infections.¹² On the other hand, the American Academy of Allergy and Immunology issued a position statement proposing that the association between inhaled steroid treatment and severe infective complications does not appear to be warranted.²⁴ Children with asthma treated with inhaled corticosteroids may exhibit growth disturbances,² and puberty may have a delayed onset in 45% of all asthmatic patients whether treated or not.²⁰ A suppressive effect of inhaled steroids on the hypothalamic-pituitary-adrenal axis (HPAA) has been demonstrated. This systemic side effect is probably dose-dependent, and it has been suggested that BDP doses of less than 14 $\mu\text{g/kg/day}$ did not cause this side effect.²⁵⁻²⁸ The local side effects observed in patients treated with BDP, oropharyngeal thrush and dysphonia, are easily eliminated by mouth rinsing or use of a spacer delivery system.²⁰

The effect of prolonged use of inhaled steroids on the immune system has not been investigated in a controlled fashion. A number of studies have investigated the effect of inhaled corticosteroids, taken in one dose²⁹ for 2³⁰⁻³² or 4^{33, 34} weeks, on the differential peripheral WBC count. These studies reported effects similar to those observed after the systemic use of steroids (i.e., an increase in WBCs and neutrophils and a decrease in eosinophils, lymphocytes, and monocytes). Blaiss et al.³⁵ showed that 24-hour treatment with inhaled BDP caused eosinopenia, lymphopenia, and neutrophil-

ia; whereas the effects on B- and T-cell numbers and on lymphocyte transformation did not show statistical significance. This study assessed the changes in the cellular immune system caused by prolonged use of inhaled corticosteroids. Our results indicate that prolonged use of BDP in children with asthma does not inhibit the studied parameters that are related to cellular immunity in daily doses of up to 600 $\mu\text{g/day}$ (or 26 $\mu\text{g/kg/day}$). The parameters measured in this study are crude and do not represent subtle potential alterations of the cellular immunity. None of the patients have shown any increased susceptibility to infections in a 2-year follow-up period. Because we investigated many unrelated biologic parameters that are notorious for their inter- and intraindividual variability, we had to use a statistical method that overcomes the fact that classical methods are not applicable.

Possible explanations for the observed findings include a minimal systemic effect of a topical pharmacologic agent, a higher sensitivity threshold of the immune system than that of the HPAA or growth systems to topical steroidal effect, and the relatively modest doses delivered to our patients.

Our findings support the idea that preventive anti-inflammatory topical steroid treatment, given in therapeutic doses, does not affect certain parameters of the cellular immunity. However, during acute asthma attacks patients may need frequent short-term treatments with oral steroids, and the effect of such treatment on the immune system is yet unknown. Further studies are needed to detect the effect of long-term treatment with higher doses of inhaled corticosteroids on the cellular immune system. We are currently in the process of prospectively investigating the longitudinal effect of long-term steroid treatment on the immune system of asthmatic children.

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