

In vitro suppression of lymphocyte activation in patients with seasonal allergic rhinitis and pollen-related asthma by citirizene or azelastine in combination with ginkgolide B or astaxanthin

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Received: September 15, 2011

Accepted after revision: February 10, 2012

Novel strategies are evaluated for management of allergic rhinitis and asthma in patients co-afflicted with both disorders. It is hypothesized that the platelet activating factor receptor antagonist Ginkgolide B (GB) and the carotenoid antioxidant astaxanthin (ASX) interact with antihistamines cetirizine dihydrochloride (CTZ) and azelastine (AZE) to potentiate their ability to downregulate potentially pathological immune activation. Peripheral blood mononuclear cells from asthmatics and healthy subjects, cultured 24 hours with 50 µg/ml phytohemagglutinin (PHA) or PHA plus each drug are analyzed by flow cytometry for expression of CD25+ or HLA-DR+ by CD3+ (T cells). Results are reported as stimulation indices for CD3+CD25+ (SICD3+CD25+) and CD3+HLA-DR+ (SICD3+HLADR+) cells in cultures treated with PHA alone, versus cultures treated with both PHA and drugs. Optimal suppression of activated cells was observed in cultures stimulated with ASX 10⁻⁶ M + CTZ 10⁻⁶ M (SICD3+CD25+, $p = 0.016$; SICD3+HLADR, $p = 0.012$); ASX 10⁻⁶ M + AZE 10⁻⁶ M (SICD3+CD25+, $p = 0.012$; SICD3+HLADR, $p = 0.015$); GB 10⁻⁶ M + CTZ 10⁻⁶ M (SICD3+CD25+, $p = 0.024$, SICD3+HLADR+, $p = 0.019$). Results demonstrate improved activity of antihistamines by 2 phytochemicals, suggesting dosing strategies for animal trials of ASX- or GB-augmented formulations for seasonal allergic rhinitis and asthma.

Keywords: T lymphocyte, astaxanthin, ginkgolide, citirizine, azelastine, allergic rhinitis, asthma bronchiale, mononuclear cells

Previous studies by authors of the present report suggest that extracts of *Ginkgo biloba* offer substantial promise to mediate minimally toxic suppression of the fundamental processes underlying immune activation and inflammation when used in combination with other drugs (1). Subsequent studies of patients suffering from allergic asthma, in our laboratory revealed that Ginkgolide B (GB), a terpene lactone component of *Ginkgo biloba* with antioxidant and PAF-inhibitory properties, affected *in vitro* activation of T lymphocytes by a pathway distinct from the conventional treatment with anti-inflammatory and/or immunosuppressive drugs (2, 3). Permanent allergic inflammation present in all patients with allergic asthma, make inhaled corticosteroids and other anti-inflammatory drugs a widely accepted first line treatment (4–

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6). Unique climatic characteristics favor high prevalence of seasonal allergic rhinitis in Kuwait (7). Consequently pollen-related asthma is one of the most common asthmatic syndromes among Kuwaitis. Second generation, non-sedating antihistamines which act by racemic-selective blockade of the H1 histamine receptor are becoming increasingly important components of treatment for these disorders (8). This is particularly true when seasonal increases in dust and pollen combine with the extreme dry conditions and intense heat of Kuwait and surrounding nations to exacerbate symptoms in afflicted persons (8). The present investigation is a pilot study for evaluation of the ability of the PAF receptor antagonist GB and the carotenoid antioxidant ASX to augment the therapeutic potential of H1 antihistamines, using expression of immune activation biomarkers by CD3+ T lymphocytes as an *in vitro* model. The authors of this report hypothesize that the aforementioned phytochemicals affect leukocyte cellular homeostasis in ways that potentiate the immunoregulatory activity of CTZ and AZE. It is anticipated that outcomes reported here will serve as a general guide for dose ratios of each compound to achieve optimal therapeutic effects in design of animal studies and ultimately in development of human clinical protocols for treatment of asthma and other syndromes characterized by pathological dysregulation of inflammatory processes.

Materials and Methods

Subjects participating in study (patients and controls)

Subjects for this study included 10 Kuwaiti patients, 6 male and 4 female, ranging in age from 21 to 35 years (mean 26 ± 1.2 years), suffering from seasonal allergic rhinitis concurrently with mild to moderate seasonal asthma. Disease duration for the study participants ranged from 0 (newly diagnosed) through 5 years. A cohort of 7 non-asthmatic subjects, 3 male and 4 female ranging in age from 21–30 years, (mean 25 ± 1.0 years) were included as case controls for this investigation. All asthma patients had been referred to the outpatient clinic of the Kuwait Allergy Center and fulfilled criteria of the International Consensus Report on Diagnosis and Management of Asthma that may be broadly defined as presence of two major pathophysiological trends involving airway obstruction and inflammation-related airway remodeling (9). Atopy was diagnosed on the basis of one or more positive skin prick tests to the local pollen allergens typical for desert region, positive specific IgE to the same allergens, and increased level of total IgE. Mean serum IgE levels for asthmatic participants in this study were 360 (185–450) IU/ml and 100 (90–120) IU/ml for the non-asthmatic control group. All patients were under treatment with anti inflammatory medication in the form of inhaled steroids at dosages prescribed by their healthcare providers. None of the patients had received systemic corticosteroids for at least 6 weeks prior to blood collection. Participation in this study was voluntary and met Kuwait University's human subject protection criteria, and it was approved by Institutional Review Board (IRB)/ethics committee. Informed consent agreements were obtained from all participating subjects.

Cell Cultures

Blood samples from 10 patients and 7 healthy control subjects were used as a basis for determination of drug effects on PHA-augmented Peripheral blood mononuclear cells (PBMC) activation for each independent assay performed. Venous blood for each subject was collected in polyethylene tubes containing EDTA during a one hour morning time interval. PBMC were separated by Ficoll-paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Cells were washed and suspended in RPMI 1640 medium (Gibco BRL,

Gaithersburg, MD) at a density of 1×10^6 cells/ml. PBMC were stimulated with 50 $\mu\text{g/ml}$ PHA (Sigma Immunopharmaceuticals, St. Louis Mo.), in the presence and absence of test drugs. Test drugs used included: cetirizine dihydrochloride (CTZ) 10^{-8} – 10^{-6} M (Pfizer Pharmaceuticals, Norwich CT); and azelastine hydrochloride (AZE) 10^{-8} – 10^{-6} M (Wallace Pharmaceuticals, Somerset NJ); astaxanthin (ASX) 10^{-8} – 10^{-6} M (Natural Alternatives International (NAI) Inc., San Marcos CA); ginkgolide B (GB) 10^{-8} – 10^{-6} M (NAI San Marcos CA). PBMC from asthmatic and non-asthmatic subjects were stimulated with combinations of one antihistamine plus either GB or ASX. Cell cultures were supplemented with antihistamines at 10^{-8} – 10^{-6} M in combination with ginkgolide B or astaxanthin each, at 10^{-8} – 10^{-6} M. Each reagent was added at the outset of a 24 hours culture period, followed by harvest of the cellular fraction for immunophenotyping studies. Phytochemical dosing strategy was based on similar data produced in earlier investigations conducted by authors of the present report (2, 3).

Flow cytometric analysis

Cells harvested from cultures by centrifugation were incubated for 30 min at 4 °C with 10 μl each of fluorescein-isothiocyanate (FITC)-CD3 and phycoerythrin (RD1)-CD25 or HLA-DR conjugated monoclonal antibodies (mAb) (Dakopatts, A/S, Glostrup, Denmark), followed by fixation with paraformaldehyde. Two-color Flow cytometry was conducted using a Beckman Coulter FC500 automated flow cytometer (Coulter Scientific, Hialeah, FL, USA). Isotypic controls for the monoclonal antibodies (mAb) used to detect antigens of interest were established for each cell preparation. Positive analysis regions for cells expressing specific surface markers were set against controls and specific binding of fluorophore-conjugated mAb was analyzed by cytofluorography according to standard methods recommended by the manufacturer. Lymphocyte subpopulations were identified by position on forward and side scatter plots and live-gated. Expression of each antigen was reported as percentage cells positive for a particular T cell subpopulation defined by expression of CD3 (T lymphocyte marker) plus CD25 or HLA-DR, plus or minus standard error.

Measurement of drug effects

The capacity of formulations evaluated in this study to suppress immune activation was measured as a stimulation index (SI), defined as the ratio of CD3+CD25+ cells or CD3+HLA-DR+ cells in each test condition to CD3+CD25+ cells or CD3+HLA-DR+ cells in cultures treated with PHA alone. Fully stimulated cultures (cells treated with 50 $\mu\text{g/ml}$ PHA) are assigned SI values of 1.00 and compared with values for CD3+CD25+ and CD3+HLA-DR+ in cultures treated with CTZ or AZE individually or in combination with ASX, GB.

Statistical analysis

Statistical analysis was performed using an independent *t*-test for comparing T cell activation in cultures treated with CTZ, AZE, ASX or GB individually versus baseline. Comparison of T cell activation in cultures treated with a combination of two drugs with cultures treated with each of the drug individually was performed using the paired *t*-test. All statistical analyses were performed using the SPSS for Windows statistical package (Norusis/SPSS, Inc.) version 16. A value of $p < 0.05$ was considered statistically significant.

Results

T lymphocyte activation

Culture of PBMC for 24 h with 50 µg/ml of the immunostimulatory lectin PHA resulted in significant activation of T lymphocytes in asthmatics measured as increased percentage of CD3+CD25+ cells or CD3+HLA-DR+ cells versus unstimulated cultures (Table I). In unstimulated cultures, CD3+CD25+ cells represented $3.5 \pm 1.4\%$ of the CD3 compartment and increased to $38.7 \pm 2.5\%$ by treatment with PHA ($P = 0.001$). CD3+HLA-DR+ cells represented $14.1 \pm 1.2\%$ of T cells at baseline, which was expanded to $25.8 \pm 3.5\%$ following PHA stimulation ($P = 0.006$). PHA-mediated expansion of activated T cells from healthy individuals was of the same order of magnitude as cells taken from blood of asthmatics. The percentage of CD3+CD25+ cells in control cultures increased from 1.81 ± 0.30 to 25.4 ± 2.7 ($P = 0.005$), while CD3+HLA-DR+ cells increased from 6.40 ± 0.8 to 24.6 ± 2.4 ($P = 0.001$).

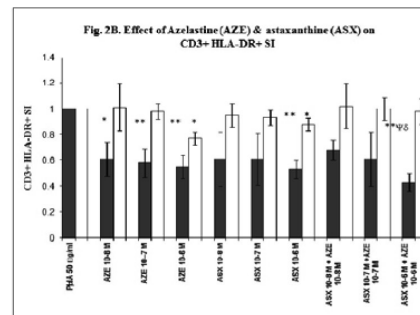
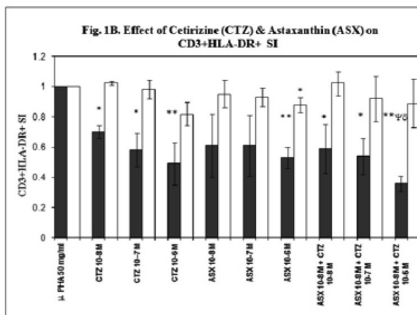
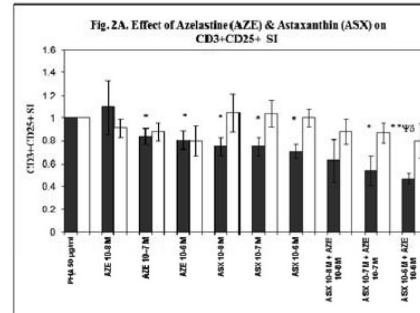
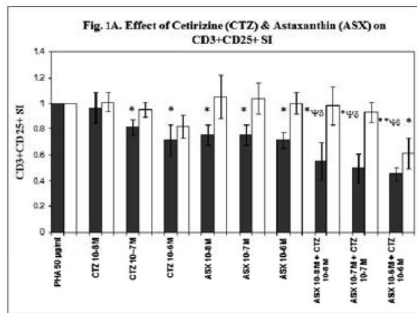
Table I. Effect of PHA stimulation on expression of immune activation antigens by T cells from blood of asthmatic patients and healthy control subjects

Lymphocyte subpopulation	Unstimulated culture	N Subjects	PHA 50 µg/ml	N Subjects	P
Asthmatic subjects					
CD3+CD25+	3.5 ± 1.4	10	38.7 ± 2.5	10	0.001
CD3+HLA-DR+	14.1 ± 1.2	10	25.8 ± 3.5	10	0.006
Control subjects					
CD3+CD25+	1.81 ± 0.30	7	25.4 ± 2.7	7	0.005
CD3+HLA-DR+	6.40 ± 0.8	7	24.6 ± 2.4	7	0.001

Cells from 10 asthma patients and 5 healthy control subjects were cultured 24 h with 50 µg/ml phytohemagglutinin (PHA). PBMC evaluated by 2-color flow cytometry were gated for CD3+ and analyzed for CD3+CD25+ or CD3+HLA-DR+ cells as a percentage of the CD3+ population. Results are reported as the mean $\pm$ SEM of % CD3+CD25+ cells or % CD3+HLA-DR+ cells in unstimulated versus fully-stimulated cultures

Effects of cetirizine (CTZ) or azelastine (AZE) on T lymphocyte activation

The ability of CTZ and AZE to inhibit PHA-induced expansion of activated human T lymphocyte subpopulations *in vitro* was evaluated over a 3-log dose range from 10^{-8} to 10^{-6} M for each drug. In cultures from asthmatics, significant suppression of PHA-induced CD3+CD25+ T cell activation was mediated by CTZ at 10^{-7} M ($p = 0.024$) and 10^{-6} M ($p = 0.032$) (Fig. 1A); and by AZE also at 10^{-7} M ($p = 0.034$) and 10^{-6} M ($p = 0.033$) (Fig. 2A). Both drugs also exhibited ability to significantly suppress PHA-induced expansion of CD3+HLA-DR+ T cells. CTZ inhibited expansion of this subpopulation at 10^{-8} M ($p = 0.019$), 10^{-7} M ($p = 0.012$) and 10^{-6} M ($p = 0.001$) (Fig. 1B); with significant inhibition by AZE seen at 10^{-8} M ($p = 0.014$), 10^{-7} M ($p = 0.009$) and 10^{-6} M ($p = 0.008$) (Fig. 2B). The effect of each antihistamine on PHA-activated cells from the non-asthmatic, control cohort was considerably less. Neither drug significantly suppressed expansion of the CD3+CD25+ subpopulation, however increases in CD3+HLADR+ were suppressed by AZE 10^{-6} M ($p = 0.02$) (Fig. 2B).



* $p < 0.05$, ** $p < 0.01$ versus baseline

Ψ $P < 0.05$ versus CTZ acting independently for a given concentration

δ $P < 0.05$ versus ASX acting independently for a given concentration

* $p < 0.05$, ** $p < 0.01$ versus baseline

Ψ $P < 0.05$ versus AZE acting independently for a given concentration

δ $P < 0.05$ versus ASX acting independently for a given concentration

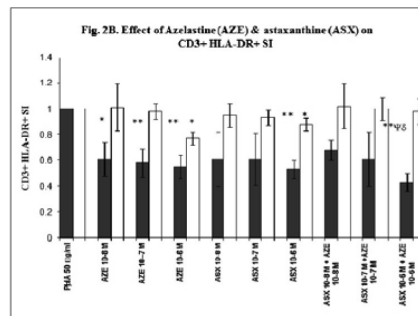
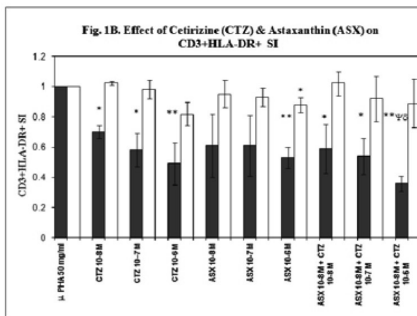
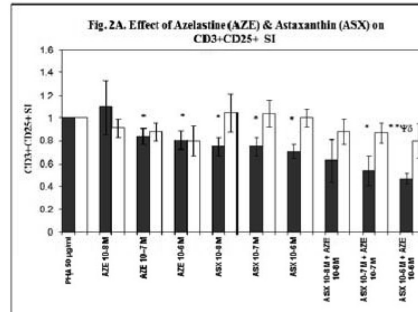
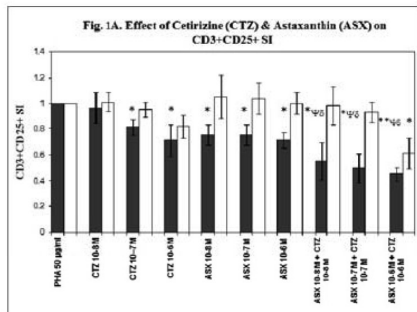
Fig. 1. Cells from 10 asthmatic patients and 7 healthy control subjects were cultured 24 hours with 50 µg/ml PHA, or PHA plus selected dosages of astaxanthin (ASX), cetirizine (CTZ) or a combination of both agents. PBMC evaluated by 2-color flow cytometry were gated for CD3+ and analyzed for CD3+CD25+ (A) or CD3+HLA-DR+ (B) as a percentage of the CD3+ population. Results are reported as stimulation indices (SI) calculated as the ratio of % CD3+CD25+ cells or % CD3+HLA-DR+ cells in fully-stimulated baseline cultures to % CD3+CD25+ or % CD3+HLA-DR+ respectively in cells treated with PHA plus ASX, CTZ or a combination of both agents

Fig. 2. Cells from 10 asthmatic patients and 7 healthy control subjects were cultured 24 hours with 50 µg/ml PHA, or PHA plus selected dosages of astaxanthin (ASX), azelastine (AZE) or a combination of both agents. PBMC evaluated by 2-color flow cytometry were gated for CD3+ and analyzed for CD3+CD25+ (A) or CD3+HLA-DR+ (B) as a percentage of the CD3+ population. Results are reported as stimulation indices (SI) calculated as the ratio of % CD3+CD25+ cells or % CD3+HLA-DR+ cells in fully-stimulated baseline cultures to % CD3+CD25+ or % CD3+HLA-DR+ respectively in cells treated with PHA plus ASX, AZE or a combination of both agents

Effects of astaxanthin and ginkgolide B on T lymphocyte activation

PHA-induced expansion of CD3+CD25+ subpopulations from asthmatics were suppressed significantly by astaxanthin (ASX) at a concentration of 10^{-8} M ($p = 0.014$); 10^{-7} M ($p = 0.012$); and 10^{-6} M ($p = 0.014$) (Fig. 1A), with significant effects by ASX on the size of CD3+HLA-DR+ populations only in cultures treated with 10^{-6} M of the compound ($p = 0.004$) (Fig. 1B). In PBMC cultures from healthy control subjects, ASX failed to significantly suppress expansion of PHA-induced CD3+CD25+ subpopulations at any of the concentrations

evaluated, but significantly inhibited expansion of CD3+HLA-DR+ cells at 10^{-6} M ($p = 0.043$) (Fig. 1B). For cultures of cells taken from blood of asthmatics, ginkgolide B (GB) significantly reduced PHA-induced increases in CD3+CD25+ populations only at 10^{-6} M ($p = 0.026$) (Fig. 3A); but significantly inhibited expansion of CD3+HLA-DR+ cells at a dosage of 10^{-7} M ($p = 0.04$); and 10^{-6} M ($p = 0.031$) (Fig. 3B). In cultures from blood of healthy control subjects, GB failed to significantly suppress induction of CD3+CD25+, but suppressed CD3+HLA-DR+ cells at 10^{-6} M ($p = 0.045$) (Fig. 3B).



* $p < 0.05$, ** $p < 0.01$ versus baseline

Ψ $P < 0.05$ versus CTZ acting independently for a given concentration

δ $p < 0.05$ versus ASX acting independently for a given concentration

* $p < 0.05$, ** $p < 0.01$ versus baseline

Ψ $P < 0.05$ versus AZE acting independently for a given concentration

δ $P < 0.05$ versus ASX acting independently for a given concentration

Fig. 3. Cells from 10 asthmatic patients and 7 healthy control subjects were cultured 24 hours with 50 µg/ml PHA, or PHA plus selected dosages of ginkgolide B (GB), cetirizine (CTZ) or a combination of both agents. PBMC evaluated by 2-color flow cytometry were gated for CD3+ and analyzed for CD3+CD25+ (A) or CD3+HLA-DR+ (B) as a percentage of the CD3+ population. Results are reported as stimulation indices (SI) calculated as the ratio of % CD3+CD25+ cells or % CD3+HLA-DR+ cells in fully-stimulated baseline cultures to % CD3+CD25+ or % CD3+HLA-DR+ respectively in cells treated with PHA plus GB, CTZ or a combination of both agents

Fig. 4. Cells from 10 asthmatic patients and 7 healthy control subjects were cultured 24 hours with 50 µg/ml PHA, or PHA plus selected dosages of ginkgolide B (GB), azelastine (AZE) or a combination of both agents. PBMC evaluated by 2-color flow cytometry were gated for CD3+ and analyzed for CD3+CD25+ (A) or CD3+HLA-DR+ (B) as a percentage of the CD3+ population. Results are reported as stimulation indices (SI) calculated as the ratio of % CD3+CD25+ cells or % CD3+HLA-DR+ cells in fully-stimulated baseline cultures to % CD3+CD25+ or % CD3+HLA-DR+ respectively in cells treated with PHA plus GB, AZE or a combination of both agents

Combined effects of ASX or GB plus cetirizine (CTZ), or azelastine (AZE) on T lymphocyte activation

Drug combinations that mediated significant suppression of PHA-stimulated values for both CD3+CD25+ and CD3+HLA-DR+ subpopulations and resulted in lower stimulation indices than those induced by any of the components independently are shown as follows:

ASX 10^{-6} M + CTZ 10^{-6} M ($SI_{CD3+CD25+}$, $p = 0.016$; $SI_{CD3+HLA-DR+}$, $p = 0.012$); ASX 10^{-6} M + AZE 10^{-6} M ($SI_{CD3+CD25+}$, $p = 0.012$; $SI_{CD3+HLA-DR+}$, $p = 0.015$); GB 10^{-6} M + CTZ 10^{-6} M ($SI_{CD3+CD25+}$, $p = 0.024$, $SI_{CD3+HLA-DR+}$, $p = 0.019$). The combination of GB 10^{-6} M + AZE 10^{-6} M with cells from asthmatics significantly suppressed the activated CD3+CD25+ subpopulation ($SI_{CD3+CD25+}$, $p = 0.040$) (Fig. 4A) but not CD3+HLA-DR+ subpopulation (Fig. 4B). The lowest concentrations of these drug combinations that resulted in significant suppression relative to of PHA alone were ASX 10^{-8} M + CTZ 10^{-8} M and GB 10^{-7} M + AZE 10^{-7} M which suppressed activation of CD3+CD25+ subpopulation ($SI_{CD3+CD25+}$, $p = 0.032$ and $SI_{CD3+CD25+}$, $p = 0.045$ respectively) with no effect on CD3+HLA-DR+ subpopulation in each case acting on cells from asthma patients.

Cell viability/toxicity evaluations

The Trypan Blue dye exclusion assay was used to determine the effect of each cell culture condition on viability of PBMC cultures. Relative to cultures not treated with drugs, cells incubated with GB, ASX or either antihistamine exhibited no obvious differences in viability during the time periods of assessment.

Discussion

The investigation described in the present report was undertaken to provide a preliminary strategy for increasing therapeutic efficacy of the antihistamines CTZ and AZE without increasing dosage to patients. Ultimately this approach may allow effective management of asthma, rhinitis and other allergic disease at lower maintenance dosage of inhaled corticosteroid. The present study begins to test this hypothesis by evaluating the capacity of astaxanthin and GB in combination with antihistamines to suppress activation of T cells in peripheral blood of asthma patients. Seasonal allergic rhinitis and intermittent asthma are frequently co-morbid and share the same pathophysiological and clinical basis as described by ARIA (Allergic Rhinitis and its Impact on Asthma) (10). The nasal airways and their closely related bronchial tree are integral parts of respiratory tract and one of the most important concepts regarding nose-lung interaction is their functional complementary (11). A new generation of non- or less-sedating antihistamines with excellent safety and efficacy profiles are emerging as first line therapies for allergic rhinitis and to manage co-morbid conditions (12). Moreover, substantial overlap in the immunopathogenesis of asthma and rhinitis suggests that treating both disorders together as a single airway disease with H1 antagonists may offer advantages in cost-effectiveness to treating each separately (7). The new generation of H1 blockers was designed to minimize the sedation and anticholinergic adverse effects that occur with older H1 receptor antagonists. In addition to their primary mechanism of H1 receptor blockade and inhibition of histamine release, these agents may act upstream of these events and affect T lymphocyte activation, ultimately influencing pathogenic processes dependent on T cells (13, 14). In this study we examine the capacity of ginkgolide B and astaxanthin to interact with CTZ and AZE in suppression of lectin-induced activation of T cells from the peripheral blood of asthmatics. Combinations of agents which

suppress activation to a greater degree than each component independently, suggest dosage conditions which may be evaluated for development of therapeutic formulations based on the combined activity of antihistamines with either of the two natural products used in this study.

The present investigation utilizes a model of T cell activation relying on lectin-mediated induction of the activation antigens CD25 (the IL-2 receptor) and the human leukocyte antigen-DR (HLA-DR). The distribution of T cell subpopulations expressing these antigens (CD3+CD25+ and CD3+HLA-DR+) in peripheral blood and affected tissue exhibit strong positive correlation with severity of a diverse range of inflammatory processes, including asthma and allergic disease (15, 16). In the present study relative representation of these immunophenotypes in peripheral blood was assessed by PHA treatment of PBMC cultures from each subject, resulting in expansion of activated T cells. The capacity of each selected drug treatment to inhibit activity of asthma/allergy-associated CD3+ species is reflected by the proliferation response of CD3+CD25+ and CD3+HLA-DR+ cells in drug-supplemented versus control cultures. Hence a reasonable assumption may be made, that conditions which affect these indicators may also impact severity of the disease. Several dosage conditions of astaxanthin (Fig. 1A, Fig. 1B) and ginkgolide B (Fig. 3A, Fig. 3B) independently inhibit PHA-induced expansion of both activated subpopulations to statistically significant degrees. The patterns of inhibition arising as a result of co-administration of antihistamines with ASX and GB provide insight as to how a dosing strategy might be structured so as to determine optimal dose ranges *in vivo*. As shown in Fig. 1, all drug combinations evaluated in the present study mediated varying degrees of capacity to suppress lectin-increased immunoreactivity. Three combinations in particular emerged as particular promising candidates for further evaluation since they caused significant suppression of both activated phenotypes beyond that mediated by any of the components acting individually. These included: ASX 10^{-6} M + CTZ 10^{-6} M (Fig. 1A, Fig. 1B); ASX 10^{-6} M + AZE 10^{-6} M (Fig. 2A, Fig. 2B) and GB 10^{-6} M + CTZ 10^{-6} M (Fig. 3A, Fig. 3B), all of which were observed in cells from asthmatic but not healthy individuals. The present study identified drug-phytochemical combinations that optimally suppressed potentially pathological immune activation, but did not characterize molecular mechanisms underlying the effects described. Nevertheless the known pharmacological mechanisms mediated by each agent used in these combinations provide some explanation for the observed effects, including possibly, the improved responses of cells from blood of asthmatics versus healthy subjects. T lymphocyte activation regulates cascades of events contributing to the pathogenesis of asthma (14–17). At the core of this process, Th2 cells in the lung express cytokines, principally IL-5, that interact with eosinophils to cause release of inflammatory mediators – especially PAF (16), resulting in events which include mast-cell sensitization, further eosinophil and lymphocyte recruitment and mucus secretion (18, 19). Hence, agents that have relatively minor effects on lymphocyte activation may ultimately cause major changes in multiple processes coordinated by T cells. Antihistamine-mediated suppression of T cell activation observed in this study may be accounted for by the fact that cellular signaling pathways which promote tissue damage in asthma exert positive feedback and increase T cell activation (16, 18). For example, the capacity of GB to attenuate T cell-dependent aspects of asthma and allergic disease may be at least partly explained by an observation made in 1992 by Calabrese et al., that activated T cells express elevated levels of the high-affinity PAF receptor (16). Thus, PAF receptor inhibitors such as GB, potentially interrupt positive feedback mechanisms by which expression of IL-5 and other asthma-associated inflammatory mediators are produced by T cells, thereby decreasing severity of atopic inflammation in a T cell-dependent manner. The

capacity of ASX to downregulate T cell-dependent features of atopic inflammation is predicted by the capacity of reactive oxygen metabolites expressed by many tissues as part of the inflammatory process to promote T cell activation with resulting increases in IL-5 expression by these cells and increased severity of inflammation (18, 19). Thus, antioxidant compounds such as ASX are expected to lower inflammation-associated oxidative stress, resulting in diminished T cell expression of IL-5 and reduced activation of these cells. Observations reported here are consistent with the aforementioned predicted effect of GB and ASX on histamine-mediated inflammation; and suggest mechanisms whereby these compounds might augment the effect of H1-inhibitory antihistamines. The foregoing analysis explores mechanisms by which drugs which inhibit release of inflammatory metabolites or their biological activity are also expected to exhibit immunosuppressive properties. Previous studies have shown that TH1 responses together with TH2 responses may act in combination to augment each other's activity to induce airway inflammation and airway hyper-responsiveness (17). Concordantly, in a mouse model of allergic asthma TH1 and TH2 cells were recruited together to the airways after sensitization with the model antigen OVA. However, TH1 cells were not only predominantly found early after the challenge; the presence of TH1 seems moreover to be a prerequisite for the recruitment of TH2 cells to the airways (17). Hence, a promising therapeutic approach may be mediated at least in part by suppression of T cell activity.

The ability of GB and ASX to combine in suppression of T cell activation may be accounted for based on the properties of each compound. PAF-inhibitory ginkgolides such as GB affect the same cellular signaling pathways that are used by calcineurin inhibitors to suppress T cell-dependent immune activation. Engagement of PAF with its complementary receptor causes the release of calcium from cellular stores, activation of calcineurin and ultimately, expression of genes contributing to immune activation in T cells (20, 21). Inhibition of this process at the level of calcineurin suppresses T cell activity but is associated with serious side effects. The process may also be inhibited by blocking PAF activity. Ginkgolides offer a desirable approach to this due to their low toxicity (22–24). Hence, their anti-inflammatory properties offer higher potential for therapeutic use. Significant contribution to tissue damage in asthma is also made by reactive oxygen species (ROS) (25), however no therapies specifically target this aspect of its pathogenesis. A logical approach to this problem is to administer an antioxidant as a therapeutic agent, in addition to drugs aimed at other features of the disease. The carotenoid ASX is an attractive candidate for this task since its antioxidant properties would be expected to reduce ROS-mediated tissue damage. Its antioxidant properties also may account for the effects reported in this study. ROS are substantially up-regulated by T lymphocytes during PHA-mediated activation; moreover blocking this enhancement with antioxidants such as glutathione radically alters the activation process, causing either increased or decreased T cell activity dependent on the ratio of ROS-inducing agents to antioxidants (26–28). Here astaxanthin provides an added advantage, since in addition to its powerful antioxidant capability it also stimulates an increase in activities of endogenous antioxidants such as glutathione (GSH) levels and superoxide dismutase (SOD) in host cells (29). The capacity of astaxanthin and ginkgolide B to cause potentially complex patterns of ROS concentration in the cultures to which the cells were exposed, may account for the observation that suppression of PHA-induced T cell activation did not exhibit clear dose-dependency with any of the drugs. If T cells are activated under several different conditions of ROS concentration, then patterns of activation and suppression would be observed at ratios of drugs which resulted in optimal ROS expression. Such optimal

conditions might occur at very low drug concentrations, or higher dosages. This analysis however is speculative and requires further investigation to confirm. A complete mechanistic picture accounting for the results of this study remains largely to be defined.

In 2004 authors of the present report reported the capacity of ASX and GB to combine with CTZ and AZE to suppress T cell activation more effectively than either antihistamine was independently capable of (30). Outcomes of the present investigation which was designed based on major results of the aforementioned 2004 study, strongly suggest that ASX and GB also augment the potency of both antihistamines. Specifically, both phytochemicals interact with CTZ and AZE to potentiate the ability of both antihistamines to downregulate T cell activation events that may contribute to pathogenesis of asthma, allergies and other disease characterized by dysregulated inflammation. These observations may indicate a potential clinical use for astaxanthin and ginkgolide B as adjuvants to antihistamines in management of asthma and allergic disease. The prospect of eventual clinical application for findings of the present study also influenced a decision to limit the dosage of drugs to a range previously demonstrated to result in significant suppression of immunoreactivity, but leaving the dose-response potential of higher doses of stimulant untested. A major criterion under which the present research is being conducted, is to minimize dosage of all components of an interacting system of several agents. Thus a major objective in the present study was to identify the minimum possible agent dosages possible for maximum combined effect. The present investigation builds on these results to further describe correlation between immunoreactivity and drug responses of cells from blood of allergy-afflicted, versus healthy individuals. It is important to emphasize that although characterization of specific molecular mechanisms responsible for enhanced immunoregulation by drug combinations was beyond the scope of the present study, novel insight into how clinical protocols might be streamlined for optimal therapeutic benefit was gained. Drug interactions described here were in many cases undoubtedly summative effects, nevertheless identifying particularly favorable ratios of agents at which such enhancement is favored, is useful in design of future human and animal trials as related studies by this laboratory have recently demonstrated (31). As shown in Table I, differences in biomarker expression response suggest differences in immunoreactivity between cells from asthmatic persons versus controls, which is anticipated based on the higher overall level of immune activation in blood of persons with allergies or asthma (13, 20, 21). For the purpose of the present report the authors choose to let these observations remain “anecdotal”, since the objective of this investigation was to identify optimal drug combinations in asthmatic and control cells separately. Conversely the present study was not undertaken as a comparison of immunoreactivity in asthmatic versus healthy individuals.

Outcomes of experiments showing that drug combinations favorably affect immunoparameters in healthy as well as allergic individuals, suggests that the phytochemical component of the formulations used here, have potential use as prophylaxis against onset of symptoms in asthmatic and allergic individuals. Unlike antihistamines, ASX and *Ginkgo biloba* extracts may be consumed as general health aids for long periods of time, since low incidence of toxicity and side effects are associated with their use; even at high dosage (31, 32, 33). Finally, it is important to qualify the author's choice of experimental model for the present investigation in the context of previous and future studies. The ultimate goal of the research initiative for which the study reported here is an element, is creation of therapeutic formulations which prevent or mitigate asthma and allergic disease based on combined activity of phytochemicals with other drugs. To maintain human clinical relevance, it is important to utilize human cells wherever possible. The authors have in fact done this in the

early stages of this initiative (2, 3, 30). Beyond relevance to eventual treatment of humans, results of human cell culture experiments also provide valid guidelines for dosage ratios in other vertebrate models, thus providing a general strategy that the authors consider to be faster and more cost-effective than use of animal blood.

Acknowledgements

This study was supported by grants from TAMOPS 4.2.2-08/1-2008-007; 4.2.1/B-09/KONV-2010-0007; 4.2.2/B-10/1-2010-0024. The manuscript was formatted and drafted by the authors. We would like to thank Stephanie C. Fox J. D. for her help in editing during the development of the manuscript.

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