In Vitro Effects of Ginkgolide B on Lymphocyte Activation in Atopic Asthma: Comparison With Cyclosporin A

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ABSTRACT—The effects of Ginkgolide B (BN52021) on in vitro activation responses of human peripheral blood mononuclear cells (PBMC) from asthmatic patients was measured using 2-channel flow cytometric analysis of activation-associated cell surface antigens or ELISA assays for cytokines known to be expressed by PBMC during T1 or T2 immunological activation. BN5201 is an anti-inflammatory extract of Ginkgo biloba and has been used therapeutically. It is a known inhibitor of platelet activating factor (PAF), which is important in the pathogenesis of asthma, and may synergise with cyclosporin A (CyA) to inhibit pathogenic immune activation in asthmatics. We compared the inhibitory effects of BN52021 and CyA (1 μM each) on activation of PBMC of asthmatic patients stimulated by phorbol myristate acetate and calcium ionophore. Inhibition of production of the cytokines IL-4 and IL-5 by BN52021 was insignificant compared to CyA. However, BN52021 significantly reversed the increase in activation-associated CD45RA expression, with a trend towards decreased expression of HLA-DR. Lymphocyte activation markers were not significantly altered by CyA. Since they appear to have differing effects on activated cells, the anti-inflammatory effects of CyA and BN52021 in atopic asthma is potentially additive. The present approach may be useful for preliminary evaluation of novel therapeutic modalities for asthma treatment.

Keywords: Asthma, Ginkgolide, Lymphocyte activation, Interleukins 4 and 5, Immunosuppression

Asthma affects 5 – 10% of the world population and has increased in incidence and severity since the early 1980s (1). It is characterized by bronchoalveolar tissue eosinophilia and increased levels of activated T lymphocytes. In the respiratory tract, released membrane-derived inflammatory mediators such as platelet activating factor (PAF) and superoxide from these cells act on lung tissue to produce the characteristic symptoms of the disease (2). Bronchoalveolar lavage (BAL) fluid contains a predominance of Th2 cytokines including interleukin (IL)-5 which may be primarily responsible the increase and activation of eosinophils (2). IL-4 also plays a critical role in asthma by increasing IgE production (3). Pathogenesis of the disease is also known to involve allergen-mediated expression of an abnormal T cell phenotype involving the co-expression of CD45RA, CD45RO, IL-2R (CD25) and HLA-DR by up to 46% of T helper cells, suggesting a transitional T cell phenotype in asthma (4). The mediator of intercellular adhesion, CD54 (ICAM-1), which normally aids in recruitment of lymphocytes to sites of inflammation, is upregulated in both its membrane-bound (5) and soluble forms (6) on vascular and bronchial endothelium of asthmatics. In severe asthma, systemic immunosuppression using corticosteroids may be necessary to interrupt the inflammatory cycle (7). To minimize the side effects or avoid the use of steroids the immunosuppressant cyclosporin A (CyA) is sometimes used (8); however, it also has adverse effects, primarily nephrotoxicity (9). Less toxic immunotherapeutic agents based on an understanding of the pathogenesis of asthma have been proposed, including Ginkgo biloba extracts (10) that have at least two potentially beneficial properties. They are known to act as a PAF-receptor antagonists (2, 11). They also contain a terpene component that scavenges superoxide anions (12), a known major contributor to tissue damage in asthma. In this investigation, immunologic parameters related to the pathogenesis of
asthma were monitored in vitro, following stimulation of human peripheral blood mononuclear cells (PBMC) from atopic asthma patients in the presence of CyA versus extract of Ginkgo biloba (BN52021), followed by analysis for expression of the T2 cytokines IL-4 and IL-5 in cell culture supernatants and percentage of cells under each stimulation condition expressing the immunologically relevant cell surface antigens CD25, CD45RA, CD54 and HLADR. Results of this study will aid in determining the usefulness of ginkgolides as therapeutic agents for asthma.

MATERIALS AND METHODS

Patients

Subjects for this study included 9 Kuwaiti patients diagnosed with atopic asthma, 4 male and 5 female, ranging in age from 19 to 32 years (mean 26 ± 1.72). Disease duration ranged from 2 to 15 years. All had been referred to the outpatient clinic of the Department of Medicine, Mubarak Hospital, Kuwait. All patients fulfilled the criteria of the International Consensus Report on Diagnosis and Management of Asthma (13). Atopy was defined on the basis of one or more positive skin prick tests to a range of 20 allergens. None of the patients had received systemic therapy for at least 6 weeks prior to blood collection. The mean serum IgE was 350 (172 – 520) IU/ml. Informed consent was obtained from every patient.

Cell cultures

Venous blood was collected in polyethylene tubes containing EDTA between 9 and 10 a.m. PBMC were separated by Ficoll-paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Cells were washed and suspended in AIM-V medium (Gibco BRL, Gaithersburg, MD, USA) at a density of 2 × 10^6 cells/ml. PBMC were stimulated with 20 nM phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin (ION) (ICN Pharmaceuticals, Inc., Costa Mesa, CA, USA). The cells were harvested 24 h later and supernatants were frozen at −70°C until use. CyA (Sigma, St. Louis, MO, USA) or BN52021 (ICN Pharmaceuticals) (1 μM each) were added at the start of culture; this concentration results in complete suppression of IL-5 expression by CyA as previously reported (14). For BN52021, a range between 1 × 10^{-9} and 1 × 10^{-4} M was previously used as this has been shown to inhibit eosinophil chemotactic response to PAF (15). No change occurred in lymphocyte activation markers with 1 × 10^{-3} and 1 × 10^{-4} M in 3 of our patients (data not shown); therefore, we adopted 1 × 10^{-4} M which produced significant changes.

Cytokine expression assays

Cytokine expression (IL-4 and IL-5) was evaluated in culture supernatants by enzyme-linked immunoabsorption assay (Amersham International plc., Buckinghamshire, UK). The detection range was 10 – 400 pg/ml and 7.8 – 500 pg/ml, respectively. Colorimetric changes proportional to supernatant cytokine concentration were measured using a model 2550 EIA reader (Bio-Rad Laboratories, Hercules, CA, USA). All assays were performed in duplicate.

Flow cytometric analysis

Two-color Flow cytometry was conducted using a Coulter Epics Profile II 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 antigens were set against isotypic 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. Isotype matched antibodies were utilized to control for nonspecific fluorescence. Briefly, peripheral blood mononuclear cells from asthmatic subjects were labeled with fluorophore-conjugated, non-specific antibody, chain-matched with the antibody to be used for a particular assay. Background values for antibodies used in assays were based on fluorescence of non-specific antibodies. Monoclonal antibodies specific for human CD25, CD45RA, CD54 and HLA-DR were purchased from Dakopatts, A/S, Glostrup, Denmark. Expression of each antigen is reported as percentage cells positive for a particular surface marker plus or minus standard error.

Statistical analysis

Statistical analysis was performed using an independent t-test. All statistical analysis were performed using the SPSS for Windows statistical package (Norusis/SPSS, Inc., Chicago, IL, USA). A value of P<0.05 was considered statistically significant.

RESULTS

Effects of CyA and BN52021 on cytokine expression

Figure 1 and Table 1 show the cytokine levels in culture supernatants produced by cells under each stimulation condition. Treatment of PBMC with PMA and ION induced significant increases in cytokine expression by cells extracted from all subjects, resulting in approximately a threefold increase in expression of IL-4 (P=0.013) and an approximate fourfold IL-5 increase (P=0.0005) when results were averaged. Presence of CyA in PMA/ION-stimulated cultures was observed to suppress expression of both cytokines (P=0.008 and P=0.0005 for IL-4 and IL-5, respectively). In contrast, BN52021 had no statistically significant
Fig. 1. In vitro cytokine expression responses by PBMC from asthmatic subjects. Expression of IL-4 and IL-5 by activated or non-activated human PBMC to cyclosporine A and BN52021. Cells were cultured with media (solid columns), 20 nM PMA + 1.0 μM ionomycin (PMA/IOM) (dark hatched columns), 1.0 μM CyA + PMA/IOM (grey columns), or 1 μM BN52021 + PMA/IOM (light hatched columns). Concentrations of IL-4 and IL-5 were determined in culture supernatants by antibody capture ELISA and expressed in pg/ml. Activated cells responded to CyA treatment by suppression of both cytokines, with a non-statistically significant trend toward suppression resulting from treatment with BN52021.

Fig. 2. In vitro expression of lymphocyte activation markers in PBMC from asthma patients. Expression of lymphocyte surface activation markers by activated or nonactivated human PBMC. Cells were stimulated with media (solid columns), 20 nM PMA + 1.0 μM ionomycin (PMA/IOM) (dark hatched columns), 1.0 μM CyA + PMA/IOM (grey columns), or 1 μM BN52021 + PMA/IOM (light hatched columns). After 24 h, flow cytometric analysis was conducted for expression of CD25, CD45RA, CD54 and HLA-DR. Results are representative of 3 independent assays and are expressed as the percentage of lymphocytes in each sample expressing a particular surface antigen.

**Effects of CyA and BN52021 on expression of lymphocyte activation markers**

As shown in Fig. 2 and Table 1, Stimulation of cultures with PMA/IOM resulted in nonsignificant increases in expression of CD25, CD54 and HLA-DR versus unstimulated controls, while CD45 was unaffected. Nevertheless, individual responses to this reagent varied: cells from 4 of the 9 subjects responded by upregulation of CD25 and HLA-DR; cells from 3 subjects showed expanded fractions of either CD25 or HLA-DR, but not both when treated with PMA/IOM; and cells from 2 subjects failed to upregulate

| Table 1. IL-4 and IL-5 expression by peripheral blood mononuclear cells and lymphocyte surface antigen expression in asthma patients |
|-------------------------|-----------------|-----------------|-----------------|-----------------|
|                       | Unstimulated   | PMA/IOM         | PMA/IOM + CyA  | PMA/IOM + BN52021 |
| IL-4 (pg/ml)          | 34.4 ± 4.5      | 87.9 ± 21.3      | 30.0 ± 4.0**    | 81.7 ± 28.1      |
| IL-5 (pg/ml)          | 92.5 ± 13.2     | 440.5 ± 72.2††   | 96.2 ± 12.9***  | 374 ± 105        |
| % CD25                | 8.07 ± 3.5      | 11.8 ± 3.5       | 9.0 ± 2.7       | 7.1 ± 2.0        |
| % CD45RA             | 64.8 ± 3.8      | 68.6 ± 4.1       | 66.9 ± 4.2      | 48.8 ± 9.5*      |
| % CD54               | 23.3 ± 4.7      | 33.1 ± 5.3       | 31.1 ± 5.3      | 27.4 ± 6.9       |
| % HLA-DR             | 27.2 ± 3.9      | 34.5 ± 6.5       | 29.5 ± 5.2      | 21.2 ± 5.3       |

Cultures of 2 × 10⁶ human PBMC were isolated from venous blood and stimulated 24 h with media, 20 nM PMA + 1.0 μM ionomycin (PMA/IOM), 1.0 μM cyclosporine A + PMA/IOM, or 1 μM ginkgolide BN52021 + PMA/IOM. Expression of IL-4 and IL-5 were measured in PBMC culture supernatants by antibody capture ELISA as described in Materials and Methods. Induction of surface antigens of interest was measured in the cellular fractions of each culture by incubation with fluorophore-conjugated monoclonal antibodies specific for CD25, CD45RA, CD54 and HLA-DR, plus isotypic controls, followed by flow cytometric analysis for expression of each marker. Results of cytokine analyses were given as pg/ml ± S.E.M., and expression of lymphocyte surface markers was reported as percentage cells positive for a particular surface marker ± S.E.M. †P = 0.013, versus unstimulated cells; ††P = 0.0005, versus unstimulated cells. *P = 0.042, versus PMA/IOM-treated cultures; **P = 0.008, versus PMA/IOM-treated cultures; ***P = 0.0005, versus PMA/IOM-treated cultures.
either CD25 or HLA-DR in response to PMA/IOM. However, cellular activation was confirmed by the substantial increase in large, probably blastic cells for all PMA/IOM-stimulated cultures (data not shown). These results indicate that for the purpose of this study, PMA/IOM served as a valid positive control stimulant for each of the variables. CyA caused no changes in cell surface activation antigen expression. CD25 and CD34 expression in cells treated with BN52021 was unaffected, but expression of CD45RA was decreased significantly ($P=0.042$) with BN52021, and a trend towards the reduction of HLA-DR expression ($P=0.067$) was seen.

**DISCUSSION**

Compared to CyA, BN52021 showed different effects on cytokine production and surface antigen expression by PBMC from asthma patients stimulated with PMA and ionomycin. CyA completely inhibited IL-4 and IL-5 production, whereas BN52021 had very little effect. BN52021, but not CyA, significantly reduced CD45RA and showed a trend towards reduced expression of HLADR. CD25, CD54 were not significantly changed with either agent. The profound effect of CyA on IL-4 and IL-5 expression is probably mediated by its direct inhibition of calcineurin (16–18). BN52021 apparently fails to effectively suppress calmodulin/calcineurin dependent expression of IL-4 and IL-5. In contrast it suppressed expression of CD45RA and also appeared to reduce the frequency of HLA-DR+ cells, in both cases, to below the values for unstimulated cells. This effect was not seen with CyA. The mechanism whereby BN52021 suppresses CD45 and HLA-DR expression is unclear. PAF-R antagonism of the ginkgolide possibly fails to reduce intracellular calcium to levels at which calcineurin would be substantially inhibited, allowing continued production of IL-4 and IL-5 (19). The pathway by which this compound exerts its effect on cellular activation probably differs from the CyA-sensitive path, especially as the latter induced widely contrasting effects on cell surface antigen expression.

The potent PAF receptor antagonist BN52021 has been shown to have beneficial clinical effects on aspects of asthma pathogenesis known to be mediated substantially by PAF (20). The mechanism by which it modulates surface antigen expression during lymphocyte activation is speculative. It may be due to selective elimination of CD45RA+ cells or a shift in the equilibrium towards CD45RO+ lymphocytes. Certain states of lymphocyte activation may be associated with enhanced dual expression of CD45RO and RA (5, 21), and the reduction of RA expression may represent a suppressive immunomodulatory effect. Circulating CD45RA+/CD45RO+/HLADR+ cells, which are increased in atopic asthma, are hypothesized to be a transitory cellular phenotype, leading ultimately to a pathogenic T cell (5). By its inhibition of CD45RA and HLA-DR, we speculate that BN52021 may partly exert its clinical effect by suppression of the development of these cells. Further studies in this area looking at co-expression of CD45 isoforms and other surface antigens modulated during cellular activation and adhesion are needed.

Very little toxicity is associated with ginkgolides (2, 10, 12); moreover, no adverse immune effects have been reported in response to these compounds. This makes them ideal candidates for treatment of inflammatory disorders such as asthma, in which substantial tissue damage occurs by immunological processes involving PAF expression and free radical production. This study suggests that BN52021 may also modulate lymphocyte activation in asthmatics, an effect which is potentially beneficial for treatment of the disease and supports the hypothesis that ginkgolides have potential for development of novel approaches to treatment of asthma. We observed a differential response to PMA/IOM for some of the surface antigens; such variations in expression are probably elements of each subject's individual immune response profile. When grouped responses of each stimulation condition are considered, it is clear that the ginkgolide BN52021 exhibits potential for modulation of immunological activity in leukocytes from asthma patients by a mode different from that of cyclosporine. This suggests that this class of drug may have potential for development as therapeutic agents in novel approaches to treatment of the disease.

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