British Journal of Pharmacology (1995) 115, 1141-1148

Modulation of mite antigen-induced immune responses by lecithin-bound iodine in peripheral blood lymphocytes from patients with bronchial asthma

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1 *Dermatophagoides farinae* (*Df*) mite antigen induced IgE synthesis associated with an imbalance of cytokine production in mite-sensitive patients with bronchial asthma; increased production of interleukin 4 (IL-4), and decreased production of interferon-γ (IFN-γ) was specifically induced in these patients' lymphocytes.

2 Lecithin-bound iodine (LBI), with which children with bronchial asthma have been successfully treated in the range of 0.5 to 5 μM, concentrations comparable to LBI blood levels in medicated individuals, modified mite antigen-induced immune responses, thereby decreasing abnormal lymphocyte functions.

3 In *Df* antigen-driven immune responses, inhibition of IgE generation accompanied by suppression of IL-4 and the recovery of IFN-γ production was successful when LBI was used *in vitro*.

4 LBI also acted on normal PBMCs by downregulating the IL-4-induced IgE synthesis, phytohaemagglutin (PHA)- and phorbol myristate acetate (PMA) plus calcium ionophore (CaI)-induced IL-4 secretion, and by upregulating purified protein derivatives (PPD)-induced IFN-γ production. Therefore, LBI was capable of inhibiting the IgE and IL-4 responses and of enhancing IFN-γ production both from allergen-stimulated atopic cells and from non-atopic cells appropriately stimulated.

5 The expression of human histocompatibility leukocyte antigen (HLA), class II antigens and intercellular adhesion molecule 1 (ICAM-1) on monocytes, crucial molecules for T cell-monocyte interactions, was not altered by LBI.

6 LBI probably acts as an immunomodulator to ameliorate mite antigen-induced abnormal cell-mediated immune responses in patients with bronchial asthma caused by *Df* antigen thereby leading to improvement of their clinical status.

Keywords: bronchial asthma; *Dermatophagoides farinae*; IgE; cytokines; interferon-γ; interleukin 4; lecithin-bound iodine; HLA class II antigen; ICAM-1

Introduction

Bronchial asthma is caused by inflammatory responses in the airway. The disease affects approximately 5% of the Japanese population and the rate of death from asthma was approximately 1% in patients treated specifically by allergologists (Matsui & Baba, 1990). The increasing incidence of childhood asthma despite advances in therapeutic strategies, necessitates the development of a therapy to fit the immunopathogenesis of the disease. Moreover, a deeper understanding of the immunological abnormalities that underly bronchial asthma is required. We studied peripheral blood mononuclear cells (PBMCs) in patients with childhood asthma focussing on mite antigen-related cell-mediated immunity.

Df antigen-specific T lymphocytes were activated *in vitro* when PBMCs were stimulated with *Df* antigen in the presence of the T lymphocyte growth factor, interleukin 2 (IL-2), in patients' lymphocytes but not in normal lymphocytes, as previously reported (Noma *et al.*, 1987; 1989; Yoshizawa *et al.*, 1989). This response should prove useful for identifying aetiological allergens and for monitoring disease activity in allergic patients, including those with bronchial asthma. *In vitro* stimulation of patients' lymphocytes with *Df* antigen also leads to polyclonal IgE generation associated with the inhibition of interferon-γ (IFN-γ) production concomitant with the overproduction of interleukin 4 (IL-4).

Lecithin-bound iodine (LBI) was prepared by the reaction of purified soybean lecithin with iodine in carbon tetrachloride. The chemical structure of LBI is shown in Figure 1 (Bhowmik *et al.*, 1967; Namba *et al.*, 1993). LBI has been shown to be clinically effective for the treatment of ocular hypersensitivity (Komoto *et al.*, 1970), thyroid diseases and childhood asthma (Nakajima *et al.*, 1974). It has been reported that clinical conditions were improved in 70.3% of 53 children with bronchial asthma during the course of one to five years' treatment with 20 to 80 nM kg^{-1} of LBI, although they could not perform a placebo-controlled study because of the long period of treatment required (Nakajima *et al.*, 1974). LBI blood levels were 0.09 to 0.17 μM with such a daily dose of LBI (Kawano *et al.*, 1993) and rose to 4 to 5 μM with a high dose (100 μM per day) of this drug (Namba *et al.*, 1994). Its mode of action has yet to be clarified. A few reports (Nakajima *et al.*, 1974; Seki *et al.*, 1979), showing that antibody formation, smooth muscle contraction by histamine, acetylcholine, bradykinin and 5-hydroxytryptamine (5-HT) and active and passive cutaneous anaphylaxis are clearly inhibited by treatment with LBI and that serum adenosine 3':5'- cyclic monophosphate (cyclic AMP) is elevated. Results of our previous study suggest that LBI contributes to the improvement of the clinical status of patients with bronchial asthma by inhibiting *Df* antigen-induced T lymphocyte proliferation assisted by IL-2 in a dose-dependent manner (Kawano *et al.*, 1993). We here report research that shows the possible mechanisms by which LBI might affect the allergic immune response in patients with childhood asthma.

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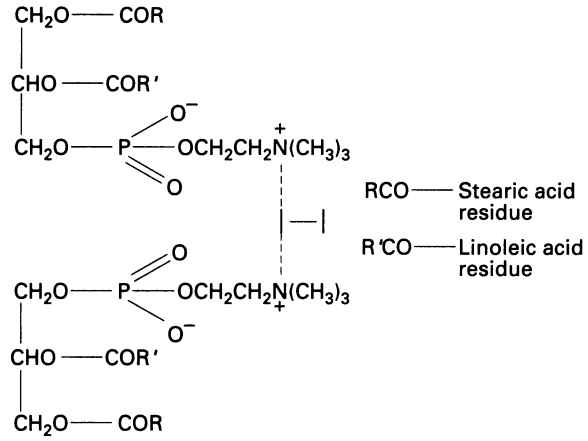

Figure 1 Molecular structure of lecithin-bound iodine (LBI).

Methods

Subjects

Twenty-four patients with bronchial asthma, 3 to 22 years of age (13.9±5.8) and 20 normal controls, aged 6 to 26 (15.1±6.5), were studied. These patients all had positive immediate skin reactions (an immediate wheal response to the skin prick test) to housedust mite. Serum IgE scores against *Df* as determined by RAST (Miyamoto *et al.*, 1974), varied from 3 to 5. The diagnostic criteria for bronchial asthma were those of the American Thoracic Society. All the patients studied were in an asthmatic attack-free state at the time of examination, and none had been given oral administrations of corticosteroids or anti-allergic agents. Informed consent was obtained from each patient.

Separation of peripheral blood mononuclear cells

Heparinized whole blood was withdrawn from healthy adult volunteers and mite-sensitive patients with bronchial asthma. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Conray density gradient centrifugation (Böyum, 1968).

Induction of IgE synthesis

PBMCs (1×10^6) were suspended in 1 ml of RPMI 1640 medium supplemented with 20 mM of L-glutamine (Sigma Chemicals Co, St. Louis, MO, U.S.A.), 5×10^{-5}M of 2-mercaptoethanol (Sigma Chemicals Co, St. Louis, MO, U.S.A.), 50 μg ml^{-1} of gentamycin (Shionogi, Osaka, Japan), and 10% foetal calf serum (FCS, Gibco, Grand Island, New York, U.S.A.) in the presence or absence of 10 μg ml^{-1} of *Df* antigen (Torii, Tokyo, Japan) or of 100 u ml^{-1} of recombinant IL-4 (Genzyme, Cambridge, MA, U.S.A.). In some experiments, rabbit anti-IFN-γ antibody (PIF-3, Hayashibara Institute, Okayama, Japan) or normal rabbit Ig was added to the cultures. The cells were cultured for 10 days, after which the culture supernatant was collected, and the IgE content was measured.

Measurement of IgE

The wells of flat-bottomed, 96-well microplates (Limbro, Mclean, Virginia) were coated with 50 μg ml^{-1} of goat anti-human IgE antibody (Cappel, Malvern, PA, U.S.A.) in 60 μl of carbonate buffer (pH 9.6). Before use, the antibody was absorbed with normal mouse Ig and normal goat Ig to avoid nonspecific binding with the mouse monoclonal antibodies and goat antibodies applied subsequently. After an overnight coating with antibody at 4°C in a humidified atmosphere, the plates were washed with phosphate-buffered saline (PBS); then the uncoated surfaces of each well were saturated with 300 μl of 0.25% gelatin (Wako, Osaka, Japan) for 3 h at 37°C. After another wash, 60 μl of a sample was placed in each well, after which the plates were incubated for 2 h at room temperature. After extensive washing of the plates, the samples were allowed to react sequentially with the following reagents: 60 μl of murine anti-human IgE monoclonal antibody (Yamasa, Choshi, Japan), 60 μl of biotinylated goat anti-mouse IgG antibody (Tago, Burlingame, CA, U.S.A.) and 50 μl of a 1000 fold dilution of streptavidin-horseradish peroxidase conjugate (BRL, Gaithersberg, MD). *o*-Phenylenediamine (BRL, Gaithersberg, MD, U.S.A.) was added, and the optical density (OD) at 490 nm was read with an enzyme-linked immunosorbent assay (ELISA) reader (APR-A4, Tosoh, Japan) and quantified by comparing it with the OD of IgE myeloma protein. The goat anti-mouse IgG antibody used as the second antibody was passed through a goat Ig- and human Ig-coupled Sepharose 4B column to prevent nonspecific binding with goat and human Ig, after which it was diluted 200 fold for use. The limit of sensitivity of the IgE assay was 40 pg ml^{-1}.

IL-4 production

PBMCs (1×10^6) were suspended in 1 ml of RPMI 1640 medium supplemented with 20 mM of L-glutamine (Sigma Chemicals Co, St. Louis, MO, U.S.A.), 5×10^{-5}M of 2-mercaptoethanol (Sigma Chemicals Co, St. Louis, MO, U.S.A.), 50 μg ml^{-1} of gentamycin (Shionogi, Osaka, Japan) and 2% human AB serum in the presence of *Df* antigen (Torii, Tokyo, Japan), 1:400 dilution of phytohaemagglutinin (PHA, Difco, Detroit, MI, U.S.A.), or 10 ng ml^{-1} of phorbol myristate acetate (PMA, Sigma Chemicals Co, St. Louis, MO, U.S.A.) plus 100 ng ml^{-1} of calcium ionophore (CaI, Sigma Chemicals Co, St. Louis, MO, U.S.A.). The cells were cultured for 2 days before the subsequent enzyme-linked immunospot (ELISPOT) assay done as described elsewhere (Hutchings *et al.*, 1989).

To detect the IL-4-producing cells, 96-well microplates (Limbro, Mclean, Virginia) first were coated with rabbit anti-IL-4 antibody (Genzyme, Cambridge, MA, U.S.A.). All the wells then were blocked with 300 μl of 0.25% gelatin (Wako, Osaka, Japan) for 3 h at 37°C, after which 1×10^5 cultured cells were incubated in the microplates in 0.2 ml of culture medium for 18 h at 37°C. After the plates had been washed three times with PBS, they were allowed to react sequentially with mouse monoclonal anti-IL-4 antibody (Genzyme, Cambridge, MA, U.S.A.) and alkaline phosphatase-conjugated goat anti-mouse Ig (Tago, Burlingame, CA, U.S.A.). After 5 more washes with PBS to remove unbound antibody, 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemicals Co, St. Louis, MO) at 1 mg ml^{-1} in 2-amino-2-methyl-1-propanol buffer was added to 0.6% agarose (BDH Chemicals Ltd, Poole, England) in the wells following a short incubation at 37°C to develop the blue-stained spots. The number of blue spots was counted under a low power inverted microscope.

IFN-γ production

PBMCs (1×10^6) were suspended in 1 ml of RPMI 1640 medium supplemented with 20 mM of L-glutamine (Sigma Chemicals Co, St. Louis, MO, U.S.A.), 5×10^{-5}M of 2-mercaptoethanol (Sigma Chemicals Co, St. Louis, MO, U.S.A.), 50 μg ml^{-1} of gentamycin (Shionogi, Osaka, Japan), and 2% human AB serum in the presence of *Df* antigen (Torii, Tokyo, Japan), 1:400 dilution of PHA (Difco, Detroit, MI, U.S.A.), 10 ng ml^{-1} of PMA (Sigma Chemicals Co, St. Louis, MO, U.S.A.) plus 100 ng ml^{-1} of CaI (Sigma Chemicals Co, St. Louis, MO, U.S.A.), or 5 μg ml^{-1} of purified protein derivatives (PPD, Japan BCG Co., Tokyo, Japan). The cells were cultured for 5 days, after which the culture supernatant was collected, and the content of IFN-γ was measured by the sandwich ELISA using rabbit (Hayashibara Inst., Okayama, Japan) and mouse monoclonal (Hayashibara Inst., Okayama,

Japan) anti-IFN-γ antibody. The limit of sensitivity of this assay was 0.1 u ml^{-1}.

FACS analysis of HLA class II and intercellular adhesion molecule 1 (ICAM-1) expression on monocytes

PBMCs (1×10^6) were cultured for two days in the presence or absence of LBI. The cells were washed extensively, stained with the monoclonal antibodies that define HLA-class II antigens or the ICAM-1 antigen, then analyzed in a fluorescence-activated cell sorter (FACS) 440 (Becton-Dickinson Electronics Lab., Mountain View, CA, U.S.A.). The monoclonal antibodies used were fluorescein isothiocyanate (FITC)-conjugated HLA-DR, FITC-conjugated Leu 10 (HLA-DQ), HLA-DP, and phycoerythrin (PE)-conjugated Leu 54 (ICAM-1), all purchased from Becton-Dickinson. In the experiments in which HLA-DP antigens were stained, cells that had been incubated with HLA-DP antibody were washed and allowed to react with $F(ab')_2$ of FITC-conjugated goat anti-mouse Ig antibody (Tago, Burlingame, CA, U.S.A.). Cells, gated on a monocyte population were analyzed in a FACS 440.

Lecithin-bound iodine (LBI)

LBI was prepared by the reaction of purified soybean lecithin with iodine in carbon tetrachloride. Its chemical structure is shown in Figure 1 (Bhowmik *et al.*, 1967; Namba *et al.*, 1993). The lecithin-iodine complex used, which is in clinical use, contains 48.2 to 50.3% lecithin iodine (LBI), approximately 10% free lecithin and about 40% phosphatidyl inositol. LBI or soybean lecithin (Daiichi Pharmaceutical Co., Tokyo, Japan) dissolved in distilled water containing 1% Tween 20 (Nakarai Chemicals LTD, Kyoto, Japan) was diluted in RPMI 1640 medium, added to the PBMCs, and cultures were started.

Statistical analysis

Students' two-tailed *t* test was used. The limit of significance was $P < 0.05$. All values are means ± s.e.

Results

Comparisons of polyclonal IgE synthesis, IL-4 secretion, and IFN-γ production in the patients' and normal individuals' PBMCs

The ability of the patients' lymphocytes to produce IgE on stimulation with *Df* antigen was examined. As shown in Figure 2a, lymphocytes from patients with bronchial asthma but not those from normal individuals, synthesized increased levels of IgE on stimulation with 10 μg ml^{-1} of *Df* antigen.

Because the induction of IgE antibody is known to be regulated by the balance between IL-4 and IFN-γ (Pène *et al.*, 1988a,b), the production of both cytokines by the patients' lymphocytes was compared with that of the normal individuals' lymphocytes. The number of IL-4-secreting cells in the patients' lymphocytes were increased by 1 μg ml^{-1} of *Df* antigen (Figure 2b). By contrast, the production of IFN-γ in the patients' lymphocytes was suppressed dose-dependently with increasing concentrations of *Df* antigen, whereas the normal lymphocytes show no significant changes in the amount of IFN-γ produced (Figure 2c).

Effect of LBI on Df *antigen-induced IgE synthesis*

LBI added to the *Df*-stimulated cultures at concentrations of 0.5 and 5 μM suppressed IgE production in a dose-dependent manner (Figure 3). In contrast to the suppressive effect of LBI, soybean lecithin (comprised of free lecithin and phosphatidyl inositol) did not inhibit IgE production. The LBI used was not toxic at these concentrations, as shown by the fact that more than 95% of the LBI-treated cells were demonstrated to be viable by the trypan blue dye exclusion method (data not shown). A high LBI concentration (50 μM), however, slightly impaired the viability of treated cells, suggesting that this drug is mildly toxic at an exceedingly high concentration.

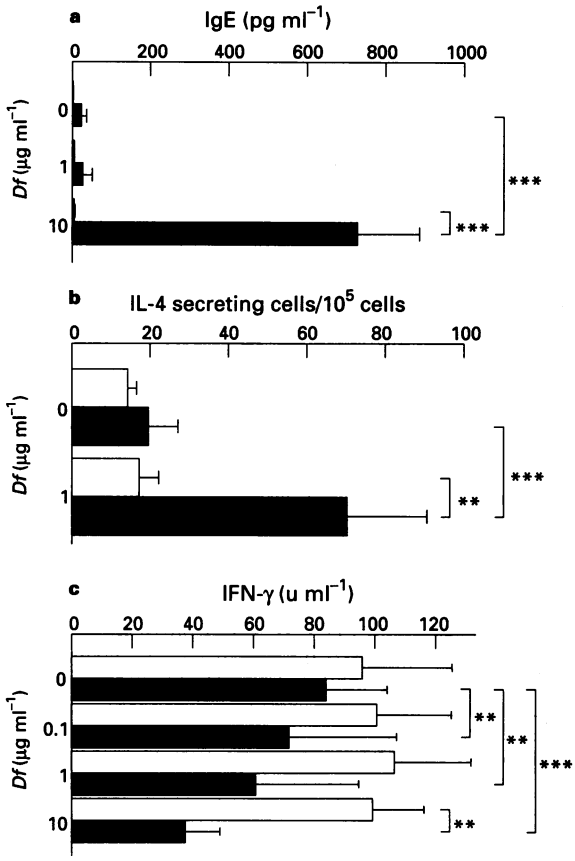

Figure 2 Comparison of polyclonal IgE synthesis, IL-4 secretion, and IFN-γ production by PBMCs of bronchial asthma patients (solid columns) and normal individuals (open columns). The PBMCs were cultured in the presence or absence of *Df* antigen, after which their abilities to produce IgE, IL-4, and IFN-γ were compared. $n = 22$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

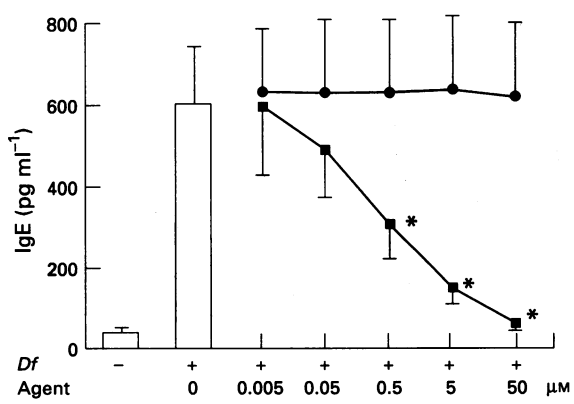

Figure 3 Effect of LBI on *Df* antigen-induced IgE synthesis. Patients' PBMCs stimulated with *Df* antigen show increased amounts of IgE as compared with unstimulated cells ($P < 0.001$). Various concentrations of LBI (■) or soybean lecithin (●) were added to the *Df*-stimulated PBMCs, and the amount of IgE released was evaluated. $n = 4$. Significant differences compared to values for the corresponding cultures with *Df* antigen only: $*P < 0.05$.

IL-4-induced IgE synthesis inhibited by LBI

To determine the effect of LBI on the generation of IgE induced by stimuli other than allergens, normal lymphocytes were stimulated with IL-4 in the presence of LBI, as IL-4 is a cytokine that induces switching to IgE (Pène *et al.*, 1988a). Similar to the inhibition of *Df*-induced IgE synthesis shown in Figure 3, IgE production was inhibited by LBI in a dose-dependent manner (Figure 4). Because LBI did not affect the amount of IgE secretion by unstimulated PBMCs, it is unlikely to function as a mitogen to induce IgE.

Effect of LBI on Df *antigen-induced IL-4 secretion*

As previous studies (Pène *et al.*, 1988a; Vercelli *et al.*, 1989) and above data clearly indicated that IL-4 is an essential factor for IgE synthesis, we investigated whether the inhibitory effect of LBI on IgE production is attributable to the suppression of IL-4 secretion. The results presented in Figure 5 show that LBI suppressed *Df*-enhancement of IL-4-secreting cell numbers, whereas soybean lecithin had no effect.

Inhibition of IL-4 production from normal lymphocytes by LBI

To determine whether the inhibition of IL-4 synthesis by LBI is specific for allergen-related immune responses, we tested the effect of LBI on the secretion of IL-4 induced by PHA, or by PMA plus CaI in normal lymphocytes. The results in Figure 6 were obviously consistent with the data in Figure 5: like *Df*-induced IL-4 secretion, PHA- or PMA plus CaI-induced IL-4 production was suppressed by LBI in a dose-dependent manner. LBI significantly inhibited the IL-4 production at concentrations of 5 and 10 μM.

Enhancement by LBI of the IFN-γ production in Df-*stimulated patients' PBMCs*

Because IFN-γ is a major cytokine suppressor of IgE production (Pène *et al.*, 1988a), the effect of LBI on IFN-γ production was examined. As expected, LBI reversed the decrease in the production of IFN-γ induced by *Df* antigen-stimulation in patients' lymphocytes in a dose-dependent manner (Figure 7). The enhancing effect of LBI on IFN-γ production was specific to PBMCs from *Df*-sensitized individuals, and was unlikely to be a mitogenic effect because unstimulated PBMCs from the same patients were not affected. In contrast to LBI, soybean lecithin, as a negative control, did not affect the IFN-γ production.

Effect of LBI on the IFN-γ production induced by PHA, PMA plus CaI, and PPD

Unlike the enhancing effect of LBI on the reduced IFN-γ production in *Df*-stimulated patients' lymphocytes, IFN-γ generation induced by PHA or PMA plus CaI in normal lymphocytes was unchanged (Figure 8). However, IFN-γ synthesis effectively induced in normal lymphocytes by PPD antigen was upregulated by LBI, albeit at a high concentration (50 μM).

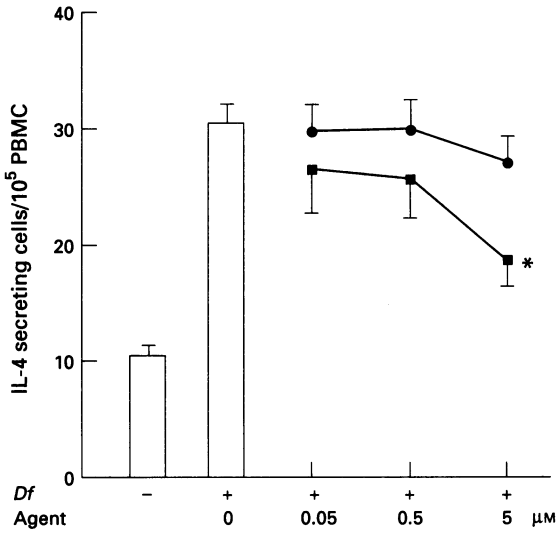

Figure 5 Effect of LBI on *Df* antigen-induced IL-4 secretion. Patients' PBMCs stimulated with *Df* antigen show increased numbers of IL-4- secreting cells as compared with unstimulated cells ($P<0.01$). Various concentrations of LBI (■) or soybean lecithin (●) were added to the *Df*-stimulated PBMCs, and the numbers of IL-4-secreting cells were quantified by the ELISPOT assay. $n=4$. Significant differences compared to values for the corresponding cultures with *Df* antigen only: $*P<0.05$.

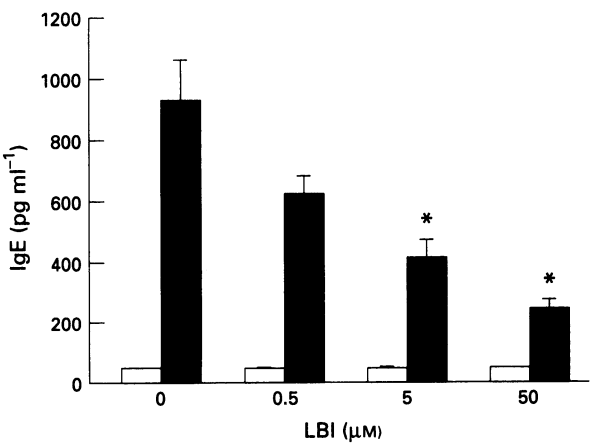

Figure 4 Inhibition of IL-4-induced IgE production by LBI. PBMCs from normal individuals, stimulated with 100 u ml^{-1} of IL-4 synthesized higher levels of IgE than untreated cells. Various concentrations of LBI were added to the PBMCs cultured with (solid columns) or without (open columns) IL-4, and the amount of IgE released was evaluated. $n=4$. Significant differences compared to values for the corresponding cultures without LBI-treatment: $*P<0.05$.

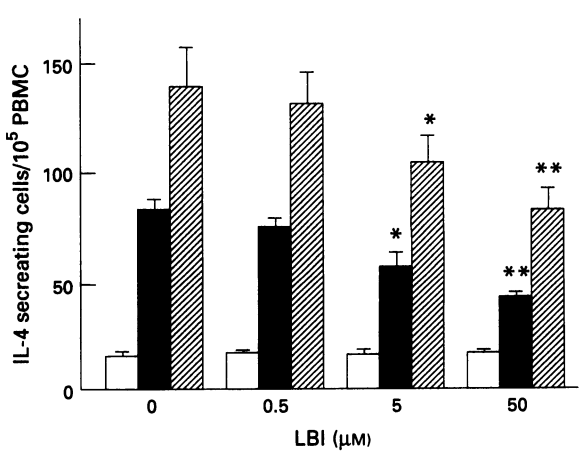

Figure 6 Suppression of IL-4 production by LBI. Normal PBMCs treated with medium (open columns), PHA (solid column) or PMA plus CaI (hatched columns) were cultured with various concentrations of LBI and then the numbers of IL-4-secreting cells were evaluated by the ELISPOT assay. $n=4$. Significant differences compared to values for the corresponding cultures without LBI-treatment: $*P<0.05$; $**P<0.01$.

Unchanged levels of LBI-induced suppression of IgE production despite the anti-IFN-γ treatment

As above data (Figure 7) clearly suggested that LBI-induced inhibition of IgE synthesis may be secondarily caused by the LBI enhancement of endogenous IFN-γ production, the effect of rabbit anti-IFN-γ antibody on the LBI-induced suppression of IgE was examined. Inhibitory activity of LBI was not abrogated by the treatment with anti-IFN-γ antibody, whereas anti-IFN-γ antibody effectively upregulated the synthesis of IgE when LBI was not added (Figure 9). As the normal rabbit Ig had no effect on these reactions, anti-IFN-γ antibody is unlikely to act as a xenoprotein.

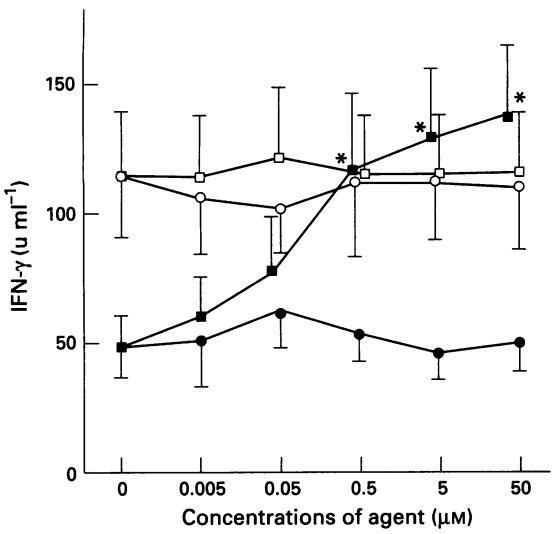

Figure 7 LBI enhancement of IFN-γ production in *Df*-stimulated patients' PBMCs. After 5 days of culture, patients' lymphocytes produced significantly reduced levels of IFN-γ upon stimulation with *Df* antigen as compared with that of unstimulated lymphocytes ($P<0.05$). Cultures treated with LBI alone (□); soybean lecithin (SL) alone (○); *Df* antigen plus LBI (■); and *Df* antigen plus soybean lecithin (●). $n=4$. Significant differences compared to values for the corresponding cultures without LBI-treatment: $^*P<0.05$.

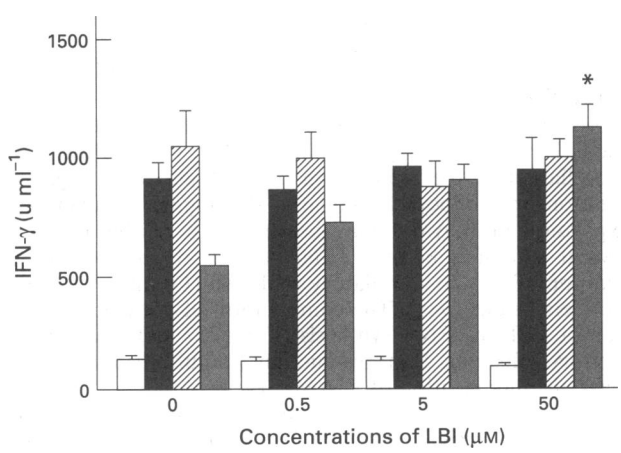

Figure 8 Enhancement of PPD-induced IFN-γ production, but not of PHA- or PMA plus CaI-induced IFN-γ production by LBI. Normal PBMCs treated with medium (open columns), PHA (solid columns), PMA plus CaI (hatched columns) or PPD (stippled columns) were cultured for 5 days in the presence of various concentrations of LBI. $n=4$. Significant differences compared to values for the corresponding cultures without LBI-treatment: $^*P<0.05$.

Effect of LBI on HLA class II antigens and ICAM-1 expression on monocytes

Our results clearly show that LBI modifies mite-antigen-related cellular responses, including those of T and B cells. The expression of HLA-class II antigens and ICAM-1 on monocytes therefore was examined because it has been suggested that these molecules on antigen-presenting cells are crucial in cell-mediated immune responses, including cytokine production (Shackelfold *et al.*, 1982; Gonwa *et al.*, 1983; Van Seventer *et al.*, 1990; Damle *et al.*, 1992). As the results presented in Table 1 show, LBI does not markedly affect the expression of HLA-DR, -DQ, -DP, or ICAM-1 on monocytes.

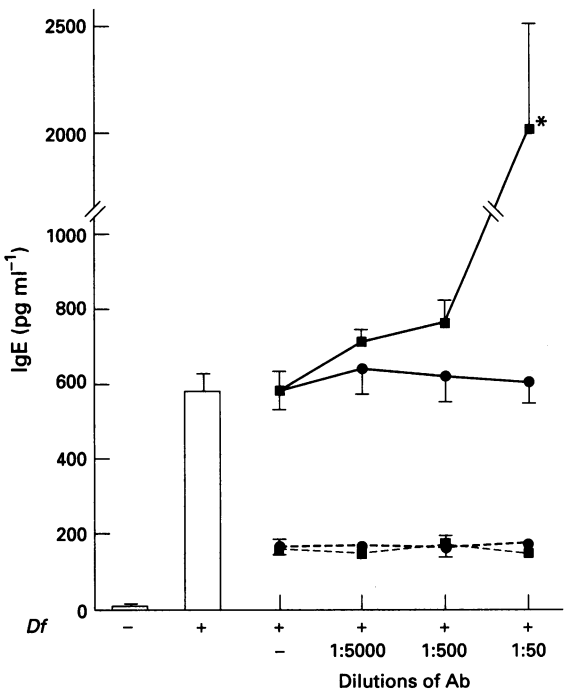

Figure 9 Effect of anti-IFN-γ antibody-treatment on LBI-induced suppression of IgE synthesis. Patients' PBMCs stimulated with *Df* antigen in the presence (- - -) or absence (—) of LBI (5 μM) were cultured with various concentrations of rabbit anti-IFN-γ antibody (■) or of normal rabbit Ig (●). $n=5$. Significant differences compared to values for the corresponding cultures without antibody-treatment: $^*P<0.05$.

Table 1 Effect of LBI on the % expression of HLA class II antigens and ICAM-1 on monocytes

	Treatment	
Antigen	*None*	*LBI*
HLA-DR	55.4 ± 13.6	58.1 ± 0.36
HLA-DQ	26.7 ± 0.51	33.4 ± 2.1
HLA-DP	17.0 ± 4.7	17.8 ± 10.7
ICAM-1	34.3 ± 11.0	39.0 ± 13.3

PBMCs were cultured for 48 h in the presence or absence of 5 μM LBI then stained with monoclonal antibodies directed against HLA class II antigens and ICAM-1. Antigen expression on the cells gated on monocytes was analysed with FACS 440. Results are means ± s.e. % of positive monocytes. For abbreviations, see text.

Discussion

The inhibitory effect of LBI on antigen-induced proliferation of peripheral blood T lymphocytes was investigated previously by Kawano *et al.* (1993). Based on findings for murine T helper lymphocytes, which show that Th1 cells preferentially induce delayed type hypersensitivity by producing IL-2 and IFN-γ (Cher & Mosmann, 1987) and that Th2 cells favour antibody formation by secreting IL-4, IL-5, and IL-6 (Boom *et al.*, 1988), and because both cell types respond to IL-2 (Kurt-Jones *et al.*, 1987), the activated human T lymphocytes induced by the allergen in atopic patients may be the counterparts of murine Th1 and Th2 cells. We speculate that the inhibitory action of LBI on *Df*-induced IL-2 responsiveness in lymphocytes from asthmatic patients (Kawano *et al.*, 1993) is evidence of inhibition of the Th1 and Th2 functions.

IL-4 generation was suppressed in a concentration-dependent manner by the addition of LBI (Figure 4). The actions of LBI on IL-4 inhibition therefore may be the secondary result of its enhancement of IFN-γ production, as shown by the regulation of Th2 cells by IFN-γ in a murine model (Gajewski *et al.*, 1988) and by the suppression of IL-4 production by IFN-γ in human lymphocytes (our unpublished data). The inhibitory actions of LBI on IL-4 synthesis were not restricted to the stimulation with *Df* antigen as shown in the suppression of IL-4 induced by PHA or PMA plus CaI (Figure 6). Notably, this suppression of IL-4 production was observed in PBMCs from non-atopic individuals. Thus the appropriate stimuli elicit the IL-4 production even in non-atopic lymphocytes. Therefore, it is concluded that IL-4 producing clones both in atopic and non-atopic individuals may similarly be inhibited by LBI, irrespective of the stumuli utilized. These findings raise the possibility that LBI could ameliorate the clinical conditions other than allergy wherein overproduction of IL-4 is pathogenic.

Unexpectedly, our study indicated that unlike IL-4, LBI potentiates the synthesis of IFN-γ, which was suppressed by stimulation with *Df* antigen in mite-sensitive asthma patients' lymphocytes (Figure 7). This IFN-γ-upregulating effect was not caused by the mitogenic action of LBI because the amounts of IFN-γ produced by unstimulated patients' lymphocytes (Figure 7) or *Df*-stimulated normal lymphocytes (our unpublished data) were not changed by the same concentrations of LBI that specifically enhanced IFN-γ production by *Df*-stimulated patients' lymphocytes. Upregulation of IFN-γ synthesis may be the direct effect of LBI, or may be caused by the inhibition of IL-4 secretion, as judged by the ability of IL-4 to suppress IFN-γ production (Peleman *et al.*, 1989; Vercelli *et al.*, 1990).

In addition to the potentiation of IFN-γ production in *Df*-stimulated patients' lymphocytes, LBI also upregulated the production of IFN-γ by normal lymphocytes stimulated with PPD, an antigen known to activate selectively human T cells of Th1 phenotype (Del Plete, 1991; Parronchi *et al.*, 1991). However, IFN-γ provoked by PHA or PMA plus CaI was not affected by this drug (Figure 8). Thus, LBI is likely to have inherent IFN-γ-potentiating activity, which may be masked in the conditions where IFN-γ was maximally synthesized by potent stimuli such as PHA or PMA plus CaI. Together with the downregulation of IL-4, upregulation of IFN-γ by LBI may facilitate the shift from Th2 to Th1. Both the IFN-γ-potentiating and IL-4-suppressing effects of LBI therefore may contribute to the inhibition of IgE synthesis on the basis of the roles of IFN-γ and IL-4 in IgE synthesis (Pène *et al.*, 1988a,b). As expected, LBI did indeed suppress the generation of IgE (Figure 3). Furthermore, IL-4-induced IgE production in lymphocytes from non-allergic individuals was equally inhibited by LBI (Figure 4). This suppression of IL-4-induced IgE synthesis is not surprising, because we have already confirmed that *Df*-induced IgE synthesis was dependent on endogenous IL-4 (our unpublished data), and the production of IL-4 in non-atopic lymphocytes was efficiently suppressed by LBI (Figure 6). Given the generation of IL-4 and IgE production in lymphocytes both from normal individuals and from atopic patients under appropriate conditions, normal lymphocytes may not be qualitatively different from atopic lymphocytes. Therefore, allergen-induced responses in atopic cells and the reactions of non-atopic cells to allergen-mimic stimulation may equally be suppressed by LBI.

However, the inhibition of IgE synthesis by LBI may not be solely dependent on the downregulation of IL-4 and upregulation of IFN-γ, since blocking of endogenous IFN-γ by anti-IFN-γ neutralizing antibody did not result in the recovery of IgE production, although IgE production in the absence of LBI was efficiently enhanced by the same concentration of the anti-IFN-γ antibody (Figure 9). Other factors known to be involved in the IgE synthesis, including IL-2 (Spiegelberg *et al.*, 1991), IL-10 (Rousset *et al.*, 1992; Aamitage *et al.*, 1993; Punnonen *et al.*, 1993b), IL-12 (Kiniwa *et al.*, 1992; Chan *et al.*, 1993; Manetti *et al.*, 1993), and IL-13 (Aversa *et al.*, 1993; Cocks *et al.*, 1993; Punnonen *et al.*, 1993a) may also be regulated by LBI.

As lecithin iodine itself is unstable in the absence of soybean lecithin, the compound used in this study could not be separated from the latter compound that contains free lecithin and phosphatidyl inositol. We therefore tested the effect of soybean lecithin, which is free of lecithin iodine, to determine the net effect of lecithin iodine on the production of IgE, IL-4, and IFN-γ. The results clearly indicate that the inhibitory effect is due to the lecithin iodine because soybean lecithin devoid of lecithin iodine did not affect IgE, IL-4, or IFN-γ synthesis.

The modifications of the *Df* antigen-related immune responses were not caused by LBI toxicity because 0.05 to 5 μM of LBI did not impair cell viability, as determined by the trypan blue dye exclusion method. These concentrations were comparable to LBI blood levels (0.09 to 0.17 μM) in individuals medicated with daily dose of LBI (20 to 80 nmol kg^{-1}) (Kawano *et al.*, 1993). Moreover, LBI blood levels reached 4 to 5 μM in normal adults medicated with high dose (100 μM/day) of this drug (Nambe *et al.*, 1994); therefore, the experimental data presented here may reflect the physiological action(s) of LBI *in vivo*.

The induction of IgE synthesis depends on HLA class II antigens (Vercelli *et al.*, 1989), and their expression and ICAM-1 expression on monocytes are critical in T cell-monocyte interactions (Shackelfold *et al.*, 1982; Gonwa *et al.*, 1983; Van Seventer *et al.*, 1990; Damle *et al.*, 1992). The effect of LBI on the expression of HLA-class II antigens and ICAM-1 on monocytes therefore was investigated. LBI, however, did not markedly affect the expression of HLA-class II antigens or ICAM-1 on monocytes (Table 1). HLA-class II antigen expression on monocytes is upregulated by IFN-γ and IL-4 (Gonwa *et al.*, 1986; Rousset *et al.*, 1988; te Velde *et al.*, 1988; Gerrard *et al.*, 1990). As shown in Figures 5, 6, 7 and 8, the fact that LBI suppressed the production of IL-4 but enhanced IFN-γ production, suggests the effects of these two cytokines on class II expression may mask each other, culminating in the cancellation of the modulation of HLA-class II antigen expression on monocytes. Similarly, the apparently unchanged expression of ICAM-1 may be the result of the actions of several cytokines because IFN-γ and IL-1 enhance the expression of this co-stimulatory molecule (Dustin *et al.*, 1986; Rothlein *et al.*, 1988). The combination of the enhancing action of LBI on IFN-γ synthesis (Figures 7 and 8) and its inhibitory effect on IL-1 production (unpublished data) may mask the modification of ICAM-1 expression.

These immunomodulatory effects of LBI differ from those of the anti-allergic agent, Tranilast, which suppresses *Df*-antigen-induced T lymphocyte proliferation as well as HLA-class II antigen expression on monocytes (Kawano & Noma, 1993).

Taken together, our results suggest that LBI acts as an immunomodulator, which favours the normalization of *Df* antigen-induced abnormal immune responses in patients with mite-sensitive bronchial asthma. *In vitro* studies described here have provided a rational explanation for its purported efficacy in asthma. Although LBI has been used to treat patients with

childhood asthma since 1962 (Sano, 1962), no severe side effects have been reported despite its usage for long periods. This drug should prove useful for patients with bronchial asthma caused by mite antigen. In addition to its beneficial effect on bronchial asthma, it may be useful for treating other atopic diseases such as atopic dermatitis as suggested by our *in vitro* data (Kawano *et al.*, 1993). The effects of LBI on cytokine production induced by causative agents, however have yet to be studied.

The lecithin-bound iodine (LBI) was provided by Daiichi Pharmaceutical Co. (Tokyo, Japan).

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(Received October 24, 1994
Revised April 4, 1995
Accepted April 7, 1995)