The effects of IPD-1151T on the expression of co-stimulatory molecules, CD40, CD80 and CD86, were investigated in vivo using mice with allergic disorders. BALB/c mice were immunized intraperitoneally with two doses of dinitrophenylated ovalbumin (DNP-OVA) at 1-week intervals. These mice then were treated intraperitoneally with 100 μg/kg of IPD-1151T once a day for 14 days, starting 7 days after the first immunization. On day 21, some mice were challenged intraperitoneally with DNP-OVA and the other mice were not challenged. All mice were autopsied on day 22 and assayed for immunoglobulin E, interleuken (IL)-4 and IL-5 productions following DNP-OVA immunization. The intraperitoneal treatment with IPD-1151T strongly suppressed immunoglobulin E contents in serum, which were enhanced by DNA-OVA immunization. IPD-1151T also caused a decrease in both IL-4 and IL-5 levels in splenic lymphocytes. We next examined the influence of IPD-1151T on co-stimulatory molecule expression on splenic lymphocytes. IPD-1151T caused suppression of CD40 and CD86 expression; however, the treatments did not affect CD80 expression.

Key words: IPD-1151T, CD40, CD86, CD80, Mouse, Splenocyte, In vivo

Introduction

IPD-1151T (suplatast tosilate) is an anti-allergic agent that suppresses several processes in the development of allergic disorders. This agent inhibits immunoglobulin (Ig)E antibody hyper-production and eosinophilia.1,2 It is also reported that IPD-1151T can suppress the production of interleukin (IL)-4 and IL-5 in response to antigenic stimulation.3

It is now recognized that two distinct signals are given in a full activation of the immune system against allergen. The first signal is provided by the ligation of the T-cell receptor and the peptide-major histocompatibility complex class II molecule complex, and the second is the interaction between co-stimulatory molecules expressed on antigen presenting cells (APC), and their ligands on T and B lymphocytes.1,5

There is much evidence that expression of the co-stimulatory molecules, CD40, CD80 and CD86, on peripheral blood leukocytes from patients with allergic disease was upregulated compared with normal subjects,6-8 suggesting the importance of these molecules in the induction and the development of the disease. However, the influences of IPD-1151T on the co-stimulatory molecule expression are poorly understood. It is generally accepted that immunization of mice with antigen absorbed to Al(OH)3 can induce IgE hyper-production and enhance the ability of lymphocyte to produce IL-4 and IL-5, which are observed in human allergic disorders. Therefore, we examined whether IPD-1151T has a suppressive activity on the expression of these molecules using mice immunized with antigen absorbed to Al(OH)3, and whether the results obtained relate to production of IgE antibody and some cytokines.

Materials and methods

Mice

BALB/c male mice, 5 weeks of age, were purchased from Charles River Japan Inc. (Atsugi, Japan).

Immunization

Ten BALB/c mice were immunized by intraperitoneal injection of 5.0 μg/ml of dinitrophenylated ovalbumin (DNP-OVA) absorbed on 4 mg Al(OH)3, and boosterd intraperitoneally with the same dose of antigen 7 days later. Five mice were intraperitoneally administered the same dose of the antigen (challenged) 21 days later.
after the first immunization and the other five mice were not (not challenged).

### Drugs and treatments

IPD-1151T was provided in a pure powder by TAIHO Co., Ltd. (Tokyo, Japan). It was dissolved in distilled water and then diluted in saline to the desired concentration. The immunized mice were intraperitoneally administered 100 μg/kg/ml per day of IPD-1151T for 14 days starting 7 days after the first immunization. The control mice received saline in the same manner.

### Assay for serum total IgE

The blood was obtained from the mice by cardiac puncture under ether anesthesia 22 days after the first immunization. After clotting, the serum was obtained and total serum IgE levels were measured using mouse IgE enzyme-linked immunosorbent assay (ELISA) kits (YAMASA Co., Ltd., Chiba, Japan) according to the manufacturer’s recommended procedures. The assay was performed in duplicate and the results were expressed as the mean (ng/ml) ± SE of five individual mice.

### Preparation of spleen cell suspension

The spleens were obtained from mice killed under ether anesthesia, and placed in RPMI-1640 medium, and pressed through a 60-gauge steel mesh to produce a single cell suspension. After centrifugation at 1000 r.p.m. for 10 min at 4°C, the pelleted cells were treated with 0.15 M Tris–0.75% NH₄Cl solution for 10 min to lyse red blood cells. After filtering through a 200-gauge steel mesh, the residual cells were washed three times and suspended in phosphate-buffered saline (PBS) at a concentration of 5 × 10⁶ cells/ml (for cytokine assay) or 1 × 10⁶ cells/ml (for flow cytometry)

### Assay for IL-4 and IL-5

IL-4 and IL-5 concentrations were assayed using mouse IL-4 and IL-5 ELISA kits (R&D system Co., Ltd., Minneapolis, MN, USA) according to the manufacturer’s recommended procedures. The ELISA was carried out in duplicate and the results were expressed as the mean (pg/ml) ± SE of five individual mice.

### Monoclonal antibodies and flowcytometry

The monoclonal antibodies (mAbs) used in this study were anti-mouse CD16/CD32 mAb, fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD40 mAb (hamster IgM), phycoerythrin-conjugated anti-mouse CD80 mAb (hamster IgG), and FITC-conjugated anti-mouse CD86 mAb (rat IgG₂a). They were purchased from PharMingen (San Diego, CA, USA). To block non-specific adherence of antibodies to murine Fcγ III/II receptors, spleen cells (1 × 10⁶/ml) were incubated with 1.0 μg of anti-mouse CD16/CD32 mAb for 10 min at 4°C, washed, and labeled with either anti-mouse CD40, CD80, or CD86 for 30 min in an ice-cold water bath. After washing once, fluorescent staining was analyzed immediately by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA, USA). Dead cells were gated out by means of forward scatter (FCS) and sideward scatter (SSC). Fluorescence of 10⁶ cells was recorded and the findings were analyzed with Consort 30 software (Becton Dickinson).

### Statistical analysis

The findings were expressed as the means ± SE and analyzed for significant differences using Wilcoxon’s test.

### Results

#### Effect of IPD-1151T on IgE antibody formation

The first experiments were carried out to examine the influence of IPD-1151T on IgE antibody formation. The results are summarized in Table 1. DNA-OVA immunization enhanced serum IgE levels as compared with normal control. Treatment of immunized mice (not challenged) with IPD-1151T caused a marked reduction in IgE levels: the level of this antibody in immunized and treated mice (not challenged) was

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**Table 1. Effects of IPD-1151T on production of IgE, IL-4 and IL-5 in BALB/c mice immunized by DNP-OVA**

| Type of mice examined | Treatment | IgE (ng/ml ± SE) | IL-4 (pg/ml ± SE) | IL-5 (pg/ml ± SE) |
|-----------------------|-----------|-----------------|-----------------|-----------------|
| Healthy control       | none      | 25.1 ± 17.4     | 12.3 ± 4.5      | 0.4 ± 0.1       |
| Non-challenged        | Saline    | 351.7 ± 73.5    | 28.2 ± 6.0      | 2.5 ± 0.5       |
| Challenged            | IPD-1151T | 32.4 ± 25.2**   | 10.8 ± 2.5*     | 0.3 ± 0.3*      |
| Challenged            | Saline    | 365.3 ± 95.0    | 39.7 ± 5.3      | 3.4 ± 0.1       |
| Challenged            | IPD-1151T | 52.9 ± 24.9*    | 22.9 ± 5.8*     | 1.0 ± 0.2*      |

Each value is the mean ± SE of five individual mice.
* Significant as compared with saline-treated mice (p<0.05).
** Significant as compared with saline-treated mice (p<0.01).
FIG. 1. Suppressive effect of IPD-1151T on co-stimulatory molecule expression on splenocytes obtained from BALB/c mice with allergic disorders. BALB/c mice were immunized intraperitoneally with two doses of 5.0 μg/ml of dinitrophenylated ovalbumin absorbed on 4 mg of Al(OH)₃ at 1-week intervals. The mice were treated intraperitoneally with 100 μg/kg of IPD-1151T for 14 days, starting after the first immunization. Splenocytes were obtained from mice 24 h after the challenge with the same dose of antigen, and assayed for co-stimulatory molecule expressions. [———], Saline-treated control mice; [——·], IPD-1151T-treated mice. Left-hand side, Non-challenged mice; right-hand side, challenged mice.
nearly identical to that in normal control (P>0.05). Moreover, the data in Table 1 also showed that treatment with IPD-1151T for 14 days caused a significant decrease in IgE contents, which was enhanced by DNA-OVA challenge immunization.

Effect of IPD-1151T on IL-4 and IL-5 production

The second set of experiments was undertaken to examine the influence of IPD-1151T on IL-4 and IL-5 production. As shown in Table 1, IPD-1151T treatment induced significant suppression of IL-4 and IL-5 contents in splenocytes, which were increased by DNA-OVA immunization. Furthermore, this suppressive effect of IPD-1151T was also observed in challenged mice (Table 1).

Effects of IPD-1151T on co-stimulatory molecule expression on splenocytes in vivo

The final set of experiments was designed to examine the influence of IPD-1151T on CD40, CD80 and CD86 expressions on splenocytes. The results are shown in Fig. 1, which is one typical profile among the results obtained from five different mice. The left-hand side of Fig. 1 shows that, in non-challenged mice, IPD-1151T markedly suppressed the third peak of CD40 (but not CD80 and CD86) molecule expression that was enhanced by DNP-OVA immunization. In contrast to the results obtained in non-challenged mice, IPD-1151T treatment in challenged mice (1, right-hand side) caused a slight decrease in fluorescence intensity of CD40. However, IPD-1151T exerted a marked suppression in the second peak of CD86 observed in challenged mice.

Discussion

It is generally accepted that antigen-specific immune responses are initiated after the collaboration of T cells with APC. Recently, it was reported that an optimal T-cell activation requires another cell-to-cell interaction. The first signal transduction is due to the interaction between the Tcell receptor and major histocompatibility complex class II molecule with an antigenic determinant. The second signal is provided by the direct contacts of co-stimulatory molecules on T cells with their ligands on APC. The signal through the binding of CD28/CTLA4 on T cells with its ligands, CD80 and CD86, on APC is a crucial co-stimulatory pathway.

Furthermore, engagement of the B-cell marker CD40 by its ligand CD40L is also recognized to play an important role in T-cell-dependent isotype switching to IgE. Clinical observations in patients with allergic disorders reveal the upregulation of co-stimulatory molecule expressions on peripheral blood leukocytes suggesting the importance of co-stimulatory molecules in the initiation of allergic diseases. In this study, we found that IPD-1151T inhibited IL-4 and IL-5 production by T helper 2 (Th2) cells and suppressed production of IgE. These findings suggested that IPD-1151T has a suppressive effect on the CD40 expression on B cells and, thereby, results in inhibition of IgE production. Moreover, the B7 markers, CD80 and CD86, are accessory molecules that play an important role in T-cell and B-cell interactions in addition to CD40. Recent studies in mice have suggested that generation of Th2 cells mainly depend on the interaction of B-cell antigen CD86 with Tcell antigen CD28. In addition, studies of human T cells have reported the importance of CD86 in Th2 cell activation, such as proliferation and cytokine secretion, in response to stimulation with allergen. In addition, it has been revealed that the expression of CD86 (but not CD80) was upregulated in allergic patients such as asthma, allergic dermatitis and allergic rhinitis compared with non-allergic subjects. Accordingly, this molecule is suggested to be an important co-stimulatory molecule in allergic responses.

Therefore, the present findings may demonstrate that IPD-1151T prevents APC and Tcell contact through the suppression of CD40 and CD86 molecule expression, and results in favorable modification of the clinical condition of allergic patients. At present, the reason why IPD-1151T administration suppressed CD40 molecule expression in non-challenged mice and CD86 molecule expression in challenged mice is unclear. However, these findings probably suggest that CD40 collaborate with CD40L prior to the binding of CD86 and CD28/CTLA4 in the antigen-specific immune responses. The observations in the present study could be available for the analysis of anti-allergic mechanisms and for the treatment of patients with allergic disorders. Additional and various studies should be carried out to clarify this problem.

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