

THE influence of an anti-allergic agent, suplatast tosilate (IPD-1151T; (\pm) -[2-[4-(3-ethoxy-2-hydroxypropoxy)phenyl-carbamoyl]-ethyl] dimethylsulfonium p-toluenesulfonate) on allergic bronchoconstriction induced by allergen and methacholine (MCh) were examined in mice. BALB/c mice were sensitized by intraperitoneal injection of dinitrophenylated-keyhole limpet hemocyanin (DNP-KLH) mixed with Al(OH)₃ (DNP-KLH). IPD-1151T was administered orally once a day for either 5 or 14 days in doses of 10, 30 or 100 mg/kg. Bronchoconstriction was measured 24 h after the final drug administration. IPD-1151T inhibited both antigen- and MCh-mediated bronchoconstriction in actively sensitized mice. The inhibition induced was closely related to the dose and frequency of oral administration of the agent. We also examined the effect of IPD-1151T on IgE production in response to DNP-KLH immunization. IPD-1151T inhibited dose-dependently both total and specific IgE concentrations in serum prepared from mice 15 days after immunization. These results strongly indicate that IPD-1151T inhibits IgE production *in vivo* and results in attenuating effect on bronchoconstriction.

Key words: Anti-allergic agent, Allergen, Bronchoconstriction, IgE, IL-4, IPD-1151T, Methacholine, Mouse, Suppression

The inhibitory effect of anti-allergic agent suplatast tosilate (IPD-1151T) on methacholine- and allergen-induced bronchoconstriction in sensitized mice

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Introduction

Bronchial hyper-responsiveness (BHR) is an important feature of bronchial asthma and the degree of BHR is believed to reflect the severity of the disease.^{1,2} The ultimate task of asthma treatment is the relief of symptoms to permit a normal style of life. The main measures to achieve this focus on the decrease of airflow obstruction and the shift of BHR towards a normal range.^{3,4} To accomplish these objectives, several types of anti-allergic drugs with bronchodilating activity have been developed and are used in the treatment and management of bronchial asthma. Although the mechanisms of BHR are not fully understood, there is an established concept that IgE is a prime mediator in development of BHR, especially allergen-induced BHR.⁴⁻⁷ That is, IgE antibodies bind to specific membrane receptors on mast cells and basophils. Combination of specific cell-bound antibody with antigen triggers a series of events leading to release of vasoactive amines and other pharmacologically active substances responsible for clinical manifestation of BHR.⁸ Recently IgE antibody receptors have also been demonstrated on a subpopulation of eosinophils and platelets, among others.⁹ Binding

of IgE antibody to receptors on these cells results in the enhancement of their function and the release of various chemical mediators responsible for allergic inflammation.¹⁰⁻¹² From these standpoints, drugs specifically inhibiting IgE antibody formation would be highly feasible for the therapy of allergic patients. However, such drugs are not available nowadays.

During immunopharmacological studies on dimethylsulfonium compounds, it is found that suplatast tosilate (IPD-1151T) suppresses Th2 type cytokine production, IgE synthesis, and chemical mediator release from mast cells in the mouse and guinea pig models of asthma.¹³⁻¹⁷ IPD-1151T has also been reported to suppress the production of Th2 type cytokines and IgE by human peripheral blood leukocytes when the cells were obtained from asthmatic patients and cultured with specific antigens in the presence of the agent.^{18,19} Furthermore, it is observed that oral administration of IPD-1151T could attenuate eosinophilia and hyperimmunoglobulinemia E in patients with Kimura's disease.²⁰ From these reports, there is a possibility that IPD-1151T may be a new anti-allergic drug that could prove useful for treatment and prevention of asthma. However, there are no reports of the influence of IPD-1151T on bronchoconstriction.

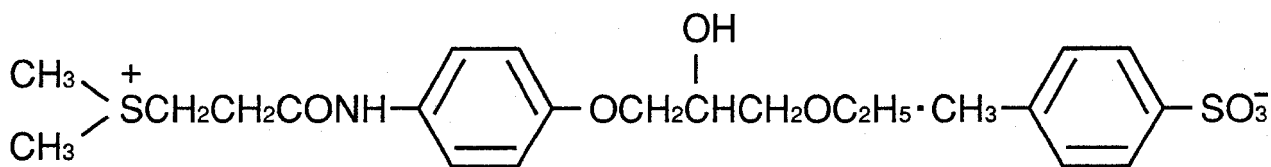


FIG. 1. Chemical structure of suplatast tosylate (IPD-1151T).

In this study, therefore, we investigated the effect of IPD-1151T on allergic airway hyper-responsiveness induced by exposure of keyhole limpet hemocyanin (KLH)-sensitized mice to repetitive antigen and to methacholine (MCh).

Materials and methods

Animals

Specific pathogen-free, normal and athymic BALB/c mice were purchased from Charles River Japan Inc., Atsugi, Japan. They were all male and 5 weeks of age at the start of the experiments. Conventional Wistar male rats were obtained from Nippon Bio-supply Center (Tokyo, Japan). All animal experimental procedures used in this study were approved by the Showa University Animal Ethics Committee, and carried out in accordance with the guidelines of the Physiological Society of Japan.

Agent

IPD-1151T (Fig. 1) was kindly supplied from Taiho Pharmaceutical Co. Ltd., Tokyo, Japan, as a preservative-free pure powder. The agent was dissolved in distilled water at 5 mg/ml just before use.

Antigens

KLH (Sigma Chemical Co. Ltd, St Louis, MO, USA) was coupled with dinitrobenzen-sulfonic acid sodium salt (DNP) (Tokyo Kasei Corp., Tokyo, Japan) according to the methods of Lee and Schon.²¹ Bovine serum albumin (BSA) (Sigma Chemical Co. Ltd.) was also coupled with DNP in a similar manner. They were abbreviated DNP-KLH and DNP-BSA, respectively.

Preparation of cell suspension

To prepare spleen cell suspension, normal BALB/c mice were killed by intraperitoneal injection with pentobarbital sodium (Abbott Lab., North Chicago, IL, USA) at a dose of 60 mg/kg. The spleen was removed, pooled from five mice and stored at 4°C until processed. The organs were pressed through a 60-gauge steel mesh and then filtered through a

200-gauge steel mesh to remove debris and cell clumps. The cells were washed five times with RPMI-1640 medium (Flow Lab., Irvine, Scotland) supplemented with 10% fetal calf serum (FCS) (Flow Lab., North Ride, Australia), 5×10^{-5} M 2-mercaptoethanol, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 100 U/ml penicillin and 100 µg/ml streptomycin (RPMI-FCS) and resuspended in the fresh medium at a concentration of 5×10^6 cells/ml. Spleen cell suspensions were also prepared from five athymic nude mice in a similar manner.

Cell culture

Spleen cell suspension (100 µl) was introduced into each well of 96-well flat-bottomed microculture plates (Nunc Intermed, Denmark), which contained 10 µg/ml of either lipopolysaccharide (LPS) or concanavalin A (Con A). The plates were maintained for 48 h in a humidified atmosphere with 5% CO₂ at 37°C. Cell activation was assessed by adding 1 µCi/well of ³H-thymidine (Amersham International plc, Bucks, UK) for the final 6 h of culture. The results were expressed as mean cpm ± SD of triplicate cultures. To prepare culture supernatant, 1 ml of spleen cell suspension was cultured in 24-well culture plates (Nunc Intermed) containing 1 ml of 10 µg/ml of Con A. After 24 h, culture supernatants were obtained after pelleting the cells by centrifugation at 2000 g for 10 min at 4°C. The supernatant was stored at -40°C until used.

Immunization

Mice were immunized by intraperitoneal injection with 5 µg/ml of DNP-KLH mixed with 2 mg of Al(OH)₃ (Wako Pure Chemical Ind., Osaka, Japan) in a total volume of 0.5 ml of saline.

Drug administration

Mice were orally administered with either 10, 30 or 100 mg/kg of IPD-1151T in a volume not exceeding 0.5 ml. Administration was performed once a day on the following schedules: one group of mice were treated for 5 days from Day 9 of sensitization and the others for 14 days from Day 0. Control mice received distilled water only in a volume not exceeding 0.5 ml.

Assay for IgE antibody

Mice were bled by cardiac puncture. Serum was isolated by centrifugation and stored at -40°C until used. Total concentration of IgE antibody in serum was assayed by commercially prepared mouse IgE enzyme-linked immunosorbent assay (ELISA) test kit (Yamasa Shoyu Co. Ltd, Chiba, Japan) according to the manufacturer's recommended procedure. Briefly, wells of a 96-well microtiter plate coated with antimouse IgE monoclonal antibody received $100\ \mu\text{l}$ of test samples and of standards, separately. After incubation at 25°C for 30 min, the wells were washed 5 times with washing buffer. Antimouse IgE enzyme conjugate ($100\ \mu\text{l}/\text{well}$) was then introduced into each well and further incubated for 30 min at 25°C . After washing, $200\ \mu\text{l}$ of substrate, tetramethylbenzidine, was dispensed, and the reaction proceeded at 25°C for 15 min. Absorbance at 450 nm was determined with an ELISA reader (MRPA4i) (Tosoh Co. Ltd, Tokyo, Japan) after adding $50\ \mu\text{l}$ of 2N HCl. The ELISA was performed in duplicate and mean absorbance was obtained. Serum antibody concentrations were calculated from standard curve and the results were expressed as mean ng/ml \pm SE of five individual mice. The concentration of DNP-specific IgE antibody in serum was assayed by passive cutaneous anaphylaxis (PCA) in Wistar rats. An intradermal injection of 0.1 ml of serum dilutions was given on the shaved back of rats. Sensitized rats were challenged by 1 mg of DNP-BSA in 1 ml of 0.5% Evans blue dye. Reciprocals of the highest dilution were recorded as the titer of the specific IgE antibody. The results were expressed as the mean PCA titer \pm SE of five individual samples.

Assay for IL-4

IL-4 contents in culture supernatants was assayed by the commercially available mouse IL-4 ELISA kit (GENZYME TECHNE Corp., Minneapolis, MN, USA) according to the manufacturer's recommendation. The ELISA was done in duplicate and the results were expressed as mean pg/ml \pm SE of five individual mice.

Measurement of airway reactivity

Twenty-four hours after the final drug administration, mice were anesthetized with intraperitoneal injection of 1.8 mg/kg body weight of pentobarbital sodium (Abbott Lab.). When an appropriate plane of anesthesia was induced, the trachea was cannulated and connected to a constant-volume respirator for small animals (SN-480-3; Shinano Seisakusho, Tokyo, Japan) that provided ventilation at a tidal volume of 0.5 ml/100 g body weight and a rate of 60 strokes/min.²¹ Under these conditions, we found that arterial oxygen tension of the mice was kept between 70 and

80 torr as measured with a transcutaneous oxygen tension meter (OXV-7101) (Nihon Kodon Co. Ltd, Tokyo, Japan). The flow at the outlet of the intratracheal cannula was measured using a differential transducer (TP-602) (Nihon Kodon Co. Ltd). One end of the differential pressure was connected to the outlet of the tracheal cannula, the other end being exposed to atmospheric pressure. The respiratory resistance was determined by the method of Konzett and Rossler²² from transpulmonary pressure, airflow and respiratory volume measures. Airway responses to KLH were measured by intravenous administration of $500\ \mu\text{g}/\text{ml}$ of KLH in a volume of 0.1 ml.²¹ In the case of examining the influence of a bronchoconstrictor agonist on airway responses, mice were injected intravenously with increasing quantities of MCh ($10^{-6}\ \text{M}$ to $10^{-2}\ \text{M}$) in a volume of 0.1 ml. The results were expressed as mean volume (ml) of air overflow \pm SE of five mice.

Statistics

Statistical significance was determined by Mann-Whitney *U*-test.

Results

Influence of IPD-1151T on airway hyper-responsiveness

The first set of experiments was designed to examine the influence of IPD-1151T on airway hyper-responsiveness. To do this, we examined firstly the minimum concentration of MCh that can cause significant

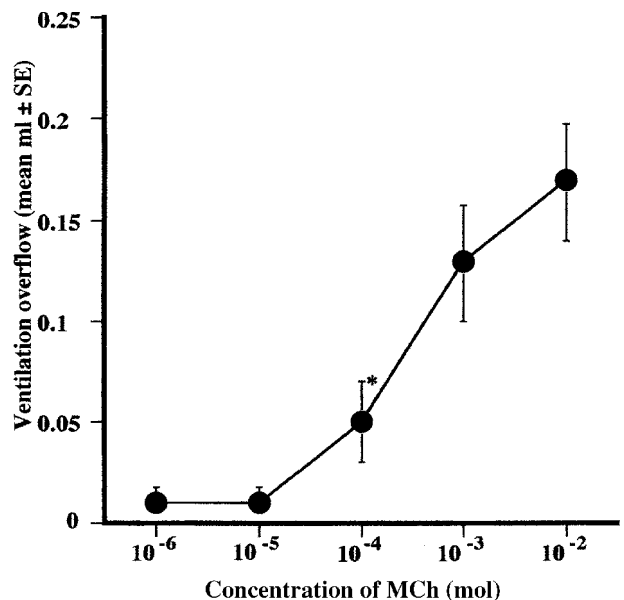


FIG. 2. Influence of MCh on bronchial responsiveness in non-sensitized mice. Each data item is the mean \pm SE of five mice.

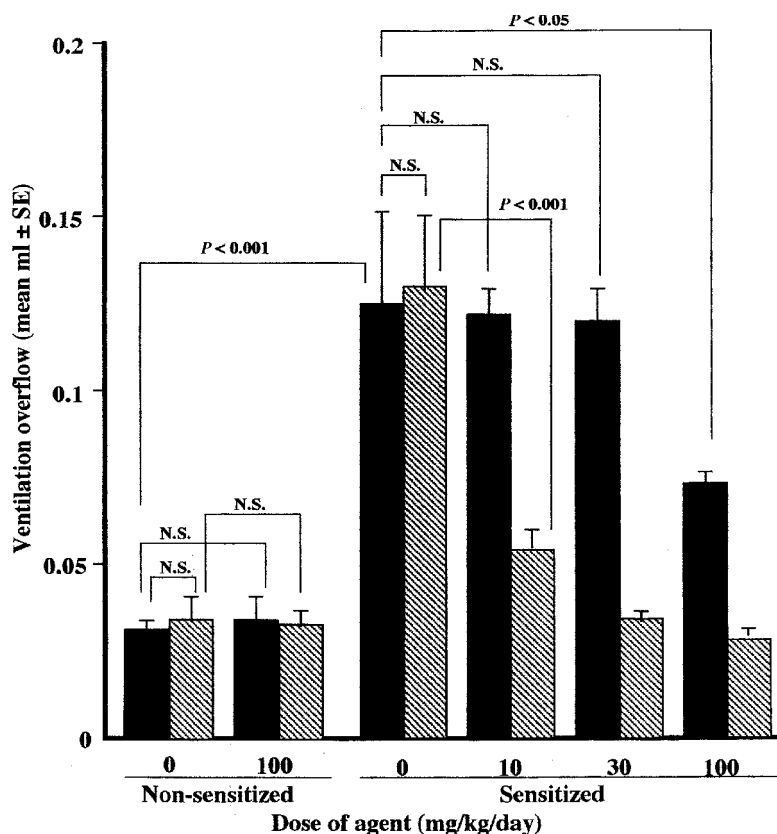


FIG. 3. Influence of suplatast tosilate (IPD-1151T) on MCh-mediated allergic bronchoconstriction in actively sensitized BALB/c mice. Mice were sensitized by intraperitoneal injection with DNP-KLH. The mice were administered orally with IPD-1151T for either 5 (starting on Day 9) (■) or 14 (starting on Day 0) (▨) days from the day of sensitization. Airway responses to 10^{-4} M MCh injected intravenously were measured on Day 15, 24 h after the final drug administration. This is one of two different experiments which gave reproducible results. N.S., Not significant.

bronchoconstriction in non-sensitized mice. The data are shown in Fig. 2. Although low doses of MCh (10^{-6} M and 10^{-5} M) scarcely affected airway responses, significant ($P < 0.05$) increase in airway overflow was observed when mice were injected intravenously with 10^{-4} M MCh. The next experiments were designed to examine the influence of oral administration of IPD-1151T on MCh-induced bronchoconstriction in DNP-KLH-sensitized mice. As shown in Fig. 3, intravenous administration of 10^{-4} M MCh into sensitized mice caused a significant increase in airway hyper-responsiveness as compared with non-sensitized control ($P < 0.001$). Oral administration of IPD-1151T inhibited MCh-induced increase in airway responses. The minimum effective dose in 5-day treatment was 100 mg/kg and that in 14-day treatment was 10 mg/kg. The influence of IPD-1151T on antigen-induced allergic airway responses is shown in Fig. 4. KLH in sensitized mice caused a strong allergic bronchoconstriction as compared with non-sensitized control ($P < 0.001$). Oral administration of IPD-1151T inhibited allergic bronchoconstriction in a dose-dependent fashion: as dose and frequency of administration were increased, volume of air overflow gradually decreased (Fig. 4).

Influence of IPD-1151T on *in vivo* IgE production

BALB/c mice were sensitized intraperitoneally with DNP-KLH. Immune serum was obtained from five individual mice 7, 10, 14, 17, 21 and 25 days after sensitization. Non-immune serum was also obtained from age-matched, non-sensitized mice. As shown in Fig. 5A, total IgE concentration in immune serum gradually increased, peaked on Day 14 and declined thereafter from Days 17 to 25. Changes in the levels of DNP-specific IgE antibody showed a similar pattern to that observed in total IgE concentration: the specific antibody was first detected on Day 7, peaked on Day 14 and declined control levels from Day 17, but sustained on Day 25 (Fig. 5B). The influence of oral administration of IPD-1151T on total and specific IgE concentration in serum prepared from mice sensitized with DNP-KLH is shown in Fig. 6. Oral administration of IPD-1151T, starting on Day 9 of sensitization, significantly inhibited both total and specific IgE concentration in serum obtained 15 days after sensitization ($P < 0.05$; Fig. 6). This inhibitory action of IPD-1151T on was further strengthened when administration of agent was started on Day 0 of sensitization (Fig. 6).

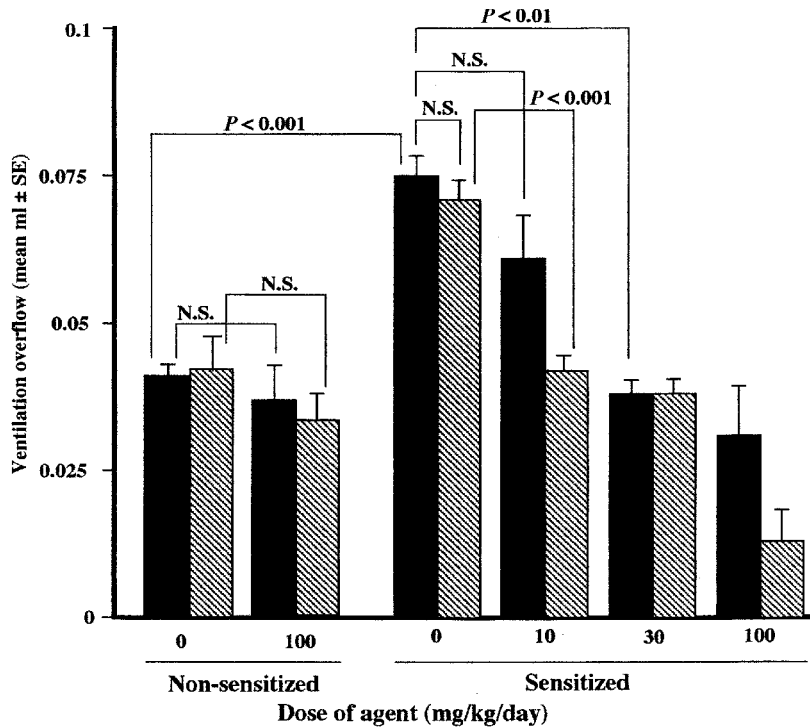


FIG. 4. Influence of suplatast tosilate (IPD-1151T) on bronchoconstriction induced by allergen in actively sensitized BALB/c mice. Mice were sensitized by intraperitoneal injection with DNP-KLH. The mice were administered orally with IPD-1151T for either 5 (starting on Day 9) (■) or 14 (starting on Day 0) (▨) days from the day of sensitization. Airway responses to 500 μ g of KLH injected intravenously were measured on Day 15, 24 h after the final drug administration. This is one of two different experiments which gave reproducible results

Influence of IPD-1151T on *in vitro* lymphocyte proliferative response and interleukin (IL)-4 production

Influence of IPD-1151T on proliferative activities of spleen cells in response to mitogenic stimulation is shown in Table 1. IPD-1151T inhibited cell prolifera-

tion in dose-dependent manner when the cells were prepared from nude mice and stimulated with LPS *in vitro*. However, IPD-1151T could not suppress T cell mitogen (Con A)-induced proliferative response of spleen cells prepared from normal mice even when the cells were cultured in the presence of 100 μ g/ml

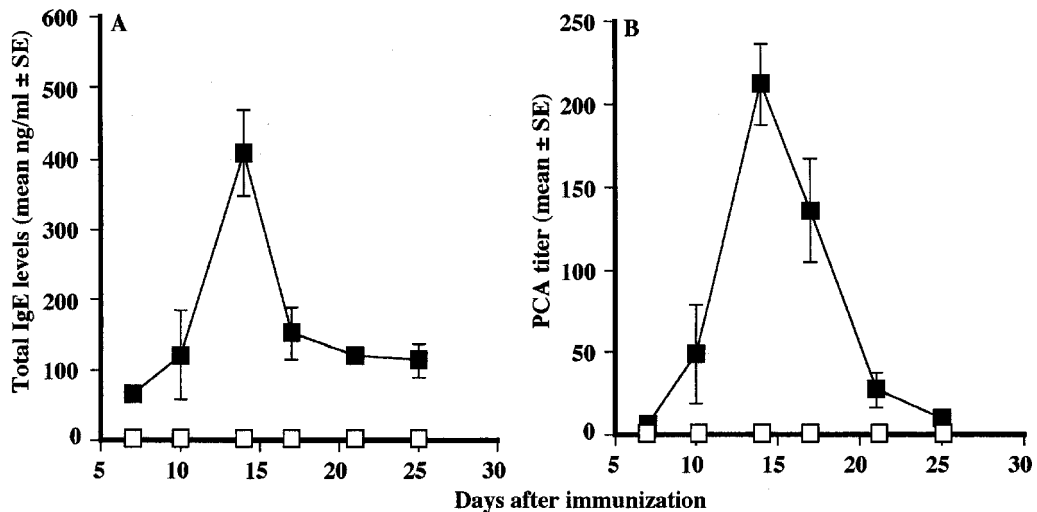


FIG. 5. Changes in total (A) and specific (B) IgE concentrations in serum prepared from BALB/c mice sensitized with DNP-KLH. The mice were injected intraperitoneally with 5 μ g of DNP-KLH mixed with Al(OH)₃ and serum was prepared from five individual mice. Each data item is the mean \pm SE of one of three different experiments which gave reproducible results. (□) Non-sensitized mice; (■) sensitized mice.

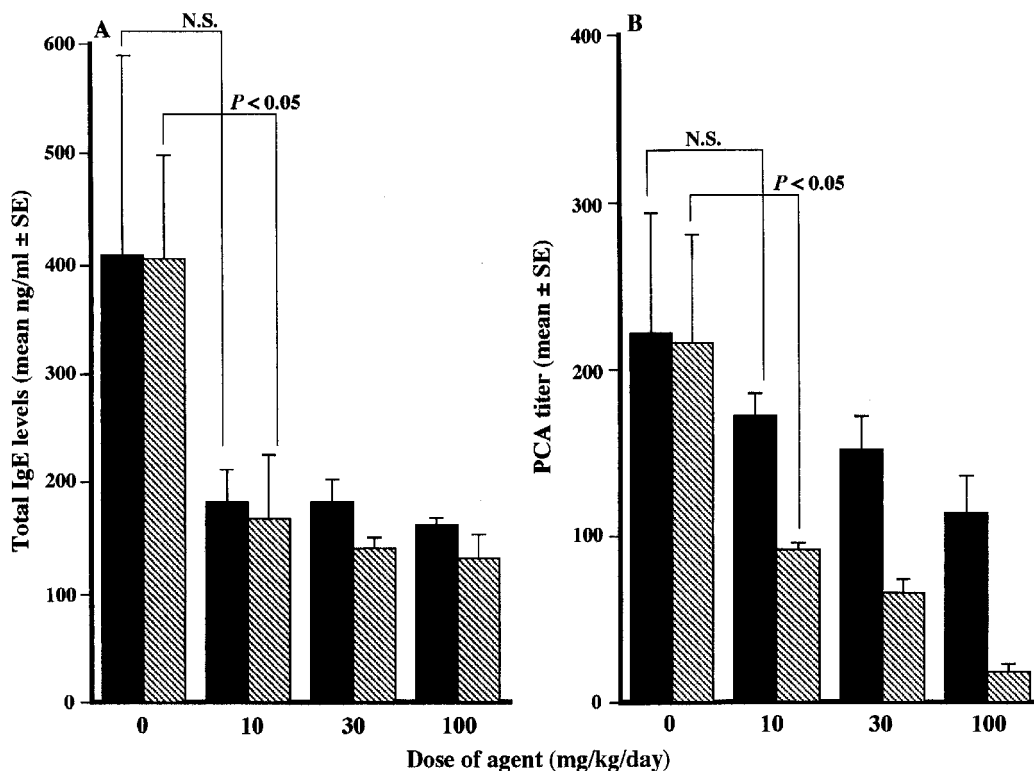


FIG. 6. Influence of IPD-1151T on total (A) and specific (B) IgE concentrations in serum prepared from BALB/c mice sensitized with DNP-KLH. All mice were injected intraperitoneally with 5 µg of DNP-KLH mixed with Al(OH)₃. The mice were administered orally with IPD-1151T for either 5 (starting on Day 9) (■) or 14 (starting on Day 0) (▨) days from the day of sensitization. Serum was prepared from five individual mice on Day 15, 24 h after final drug administration.

of the agent. IL-4 production by spleen cells in response to Con A stimulation *in vitro* was significantly inhibited by IPD-1151T. This inhibitory effect on IL-4 production was dose-dependent and first noted at a concentration of as little as 5 µg/ml (Table 2).

Discussion

The present study shows that IPD-1151T inhibits MCh-induced increase in airway responses in pre-

sensitized mice. IPD-1151T has an ill-defined mode of action, and various elements could account for this protective effect. Functional antagonism towards MCh-induced bronchoconstriction does, however, not account for the findings in the present study, since the MCh responsiveness in normal control animals is not altered (Fig. 3). It is reported that mouse has airway contractile tissue that responds when exposed to muscarinic agonists such as MCh by the vascular route.²³ There is also evidence that airway response to muscarinic agonists is presumably

Table 1. Influence of IPD-1151T on blastic activity of spleen cells in response to mitogenic stimulation^a

Dose of agent (µg/ml)	Blastic activity (mean cpm × 10 ³ ± SE)	
	Nude mice (LPS stimulation)	Normal mice (Con A stimulation)
0	25.5 ± 0.5	79.6 ± 1.3
5	16.5 ± 2.0	85.0 ± 5.3
10	14.3 ± 0.2	71.9 ± 14.7
50	9.4 ± 0.2	76.7 ± 1.6
100	6.4 ± 0.1	82.2 ± 2.2

^a Spleen cells were prepared from normal (+/+) and athymic nude (-/-) mice, and used as target cells for LPS and Con A stimulation, respectively. Cells (5 × 10⁵ cells/200 µl) were cultured in triplicate in the presence of 5 µg of mitogens for 48 h.

Table 2. Influence of IPD-1151T on *in vitro* IL-4 production by spleen cells in response to Con A stimulation^a

Dose of agent (µg/ml)	IL-4 content (mean pg/ml ± SE)	Reduction (%)
0	214.7 ± 10.4	
5	186.9 ± 27.1*	30.4
10	127.9 ± 16.1**	40.4
50	128.3 ± 7.9**	40.2
100	109.1 ± 12.5**	49.2
500	68.2 ± 12.2**	68.2

^a Spleen cells (5 × 10⁶ cells/ml) prepared from five normal BALB/c mice were cultured with 10 µg/ml Con A for 24 h. IL-4 contents in culture supernatants were assayed by ELISA.

*Not significant (P>0.05) as compared with control, **Significant (P<0.01) as compared with control.

mediated by specific muscarinic receptor activation.²⁴ Therefore, the data in Fig. 3 may be interpreted as showing that IPD-1151T inhibits muscarinic receptor activation that is induced by sensitization with DNP-KLH and results in therapeutic effects on KLH-induced increase in airway response to MCh.

The importance of IgE-related reactions in terms of their ability to influence airway function has been apparent from clinical and experimental studies. Sensitization of mice by inhalation of allergen caused not only IgE hyper-production but also increased airway reactivity.^{4,5} Histological examination of the nose and lungs including lower and upper airways prepared from sensitized mice revealed an absence of neutrophils, eosinophils and mast cells at the time of the increased airway hyper-responsiveness.⁴ It is also reported that passively transferred rabbit serum containing allergen-specific IgE is capable of sensitizing naive rabbits so that they develop airway obstruction,²⁵ as well as increased airway hyper-responsiveness after exposure to specific allergen.^{26,27} These reports strongly suggest that alterations in airway function such as hyper-responsiveness to allergens are closely linked to the presence of allergen-specific IgE. The present study revealed the inhibitory effect of IPD-1151T on levels of both total and specific IgE when the mice were administered orally with the agent (Fig. 6). It is also showed that inhibitory effect of IPD-1151T on IgE production is due to its suppressive action on B-cell proliferation (Table 1) and IL-4 production from T-cells (Table 2). Taken together, a second possible explanation for the protective effect of IPD-1151T on antigen-induced airway hyper-responsiveness (Fig. 3) would be an effect on IgE production.

Upon antigenic stimulation, cross-linking of surface IgE bound to receptors expressed on mast cells and basophils caused secretion of substances stored in granules such as histamine and serotonin, etc.^{28,29} and newly produced physiologically active substances such as leukotrien and platelet activating factor.^{30,31} These chemical mediators are reported to provoke alteration of smooth muscle in airways and result in bronchoconstriction.^{32,33} Matsuura *et al.*¹⁵ revealed the inhibitory action of IPD-1151T on degranulation of mast cells in response to antigenic stimulation *in vitro*. From these observations, there is a possibility that IPD-1151T may function as a membrane stabilizer and attenuate bronchoconstriction induced by MCh and specific antigen.

Since mice administered IPD-1151T for 14 days (100 mg/kg/day) did not show weight loss, ruffled fur and a hunched posture, compared with the control, IPD-1151T could be expected to develop into a new anti-asthmatic drug.

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