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Effect of TEI-9874, an inhibitor of immunoglobulin E production, on allergen-induced asthmatic model in rats

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Abstract

As TEI-9874, 2-(4-(6-cyclohexyloxy-2-naphtyloxy)phenylacetamide)benzoic acid reduces allergen-specific immunoglobulin E (IgE) production by human peripheral blood mononuclear cells in vitro, we evaluated its potency on an allergen-induced asthmatic model in Brown–Norway rats. Inhaled ovalbumin induced the immediate-phase asthmatic response, the late-phase asthmatic response, the infiltration of leukocytes into bronchoalveolar lavage fluid, and an increase of serum anti-ovalbumin IgE. These parameters were suppressed by the treatment with TEI-9874 (3, 10, and 30 mg/kg p.o.). The ovalbumin-induced airway hyperresponsiveness was prevented by TEI-9874 (30 mg/kg p.o.). Furthermore, the suppression of the immediate-phase asthmatic response and the late-phase asthmatic response by TEI-9874 was almost completely extinguished by the exogenous administration of rat anti-ovalbumin antiserum. These results indicate that the efficacy of TEI-9874 on the asthmatic response is mainly mediated by the suppression of allergen-specific IgE production and TEI-9874 appears to be a good candidate as therapy for IgE-mediated allergic asthma.

Keywords: Asthma; TEI-9874; Immunoglobulin E

1. Introduction

Mast cells are considered to be important conductor cells in the pathogenesis of allergic disorders, including allergic asthma (Peters, 1990; Broide et al., 1991; Schulman, 1993; Richard, 1993). Activated mast cells produce inflammatory mediators such as histamine, leukotrienes, and neutral proteases including tryptase, chymase, cathepsin G, etc. (Holgate et al., 1988; Peters et al., 1989; Sommerhoff et al., 1989; Peters, 1990). These mediators are thought to be responsible for the mucosal edema (Evans et al., 1989; Tokuyama et al., 1991) and the immediate bronchoconstriction in the immediate-phase asthmatic response. Activation of the mast cell is induced via aggregation of immunoglobulin E (IgE) receptors (FcεRI). Therefore, IgE plays an important role in allergic responses.

On the other hand, the role of IgE in the late-phase asthmatic response to inhaled allergen is less certain. The late-phase asthmatic response, which is characterized by airway narrowing occurring three or more hours after allergen provocation, is associated with epithelial cell injury and mucosal edema (Ohrui et al., 1992) that lead to airway hyperresponsiveness to non-specific stimulation. It has been reported that a humanized murine monoclonal antibody directed to the FcεRI-binding domain of human IgE, which inhibits the binding of IgE to its receptor, suppresses the immediate-phase asthmatic response and the late-phase asthmatic response to inhaled allergen in allergic asthmatic subjects (John et al., 1997; Boulet et al., 1997). These reports imply that the targeting of IgE might be a useful treatment for allergic asthma.

Brown–Norway rats are better producers of allergen-specific IgE than other strains of rats (Murphy et al., 1974; Pauwels et al., 1979; Abadie and Danon, 1980; Waserman et al., 1992; Imaoka et al., 1993). This animal demonstrates biphasic asthmatic responses, infiltration of inflammatory cells into the respiratory tract, and airway hyperre-

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sponsiveness (Eidelman et al., 1988; Sapienza et al., 1990; Elwood et al., 1991; Waserman et al., 1992; Renzi et al., 1993; Martin et al., 1993; Nagase et al., 1995; Haczku et al., 1995) to inhaled allergen. Regarding these characteristics, this animal model is very similar to allergic asthma in human and is very useful for the evaluation of anti-asthmatic agents.

TEI-9874, 2-(4-(6-cyclohexyloxy-2-naphtyloxy)phenylacetamide)benzoic acid, demonstrated strong suppression of IgE production by human peripheral blood mononuclear cells in vitro, with an IC_{50} of 27 nM, in comparison with its efficacy for immunoglobulin G (IgG) with an IC_{25} of more than 100 nM. To evaluate the effect of a reduction in allergen-specific IgE production on the immediate-phase asthmatic response and the late-phase asthmatic response to inhaled allergen, we examined the action of TEI-9874 in a Brown–Norway rat allergic asthmatic model.

2. Materials and methods

2.1. Materials

The reagents used in this study and their sources were the following: Ficoll-Paque from Pharmacia, Uppsala, Sweden; fetal calf serum from HyClone Lab., Logan, UT; human recombinant interleukin-4 from Amgen Biologicals, Thousand Oaks, CA; human recombinant interleukin-10, mouse monoclonal anti-human CD40 antibody (B-B20), and alamar blue from BioSource International, Camarillo, CA; alkaline phosphatase-conjugated extravidin, *p*-nitrophenyl phosphate disodium, and ovalbumin (Grade III) from Sigma, St. Louis, MO; $Al(OH)_3$, *Bordetella pertussis* inactive microorganism suspension, acetyl-β-methylcholine chloride, NaN_3, Tween 20, and H_2SO_4 from Wako, Osaka, Japan; rabbit anti-human IgE antibody from ICN Biomedicals, Lisle, IR; biotin-conjugated goat anti-human IgE antibody from Kirkegaard and Perry Lab., Gaithersburg, MD; human IgE from Behringwerke, Marburg, Germany; goat anti-human IgG antibody and alkaline phosphatase-conjugated goat anti-human IgG antibody from Cappel Lab., West Chester, PA; human IgG from Kent Labs, Kent, WA; biotin, avidin–biotin–horseradish peroxidase, and 3,3′5,5′-tetramethylbenzidine microwell peroxidase substrate solution from Funakoshi, Hiroshima, Japan; mouse monoclonal anti-rat IgE antibody (MARE-1), mouse monoclonal anti-rat immunoglobulin G_1 (IgG_1) antibody (MARG1-2), and mouse monoclonal anti-rat immunoglobulin G_{2a} (IgG_{2a}) antibody (MARg2a-1) from Zymed Laboratories, San Francisco, USA; bovine serum albumin from Nacalai Tesque, Kyoto, Japan; phosphate-buffered saline (PBS) and RPMI-1640 from Gibco BRL, Life Technologies, Rockville, MD, USA; Diff-Quick stain from International Reagents, Kobe, Japan; TEI-9874 from Teijin Pharmaceutical, Hino, Japan. The other reagents were obtained from Sigma.

2.2. Production of immunogloblins from human peripheral blood mononuclear cells in vitro

Immunogloblins were produced from human peripheral blood mononuclear cells as follows (Uejima et al., 1995): briefly, human peripheral blood mononuclear cells were obtained by Ficoll-Paque density gradient centrifugation from a normal volunteer. Peripheral mononuclear cells (2×10^6 cells/ml) were cultured in flat-bottomed 96-well plates (Coastar, Cambridge, MA) in a final volume of 200 μl of RPMI-1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin. Human recombinant interleukin-4 (0.1 μg/ml), interleukin-10 (0.2 μg/ml), and mouse monoclonal anti-human CD40 antibody (2 μg/ml), with or without TEI-9874, were added to the cultures on day 0. After 2 weeks, culture supernatants were harvested, and human IgE and IgG levels were determined by enzyme-linked immunosorbent assay. Suppression (%) was calculated with the following equation: (level without TEI-9874 − level with TEI-9874)/level without TEI-9874 × 100. After removal of the medium, alamar blue (20 μl/well) was added to the cultures, and incubation was carried out for 2 h. Cell viability was measured by counting the number of fluorescent (viable) cells (excitation $\lambda = 530$ nm, emission $\lambda = 590$ nm). Suppression (%) was calculated as described above.

2.3. Animals and sensitization

Brown–Norway rats (140–190 g, Charles River, Tokyo, Japan), 8 weeks of age, were used. Active sensitization against ovalbumin was performed by subcutaneous injection of 1 ml of sterile solution containing 1 mg ovalbumin and 200 mg $Al(OH)_3$ in saline; at the same time 1 ml of *B. pertussis* vaccine containing 6×10^9 heated-killed bacilli was injected intraperitoneally (day 0). On the 14th day after the first sensitization, a secondary sensitization was carried out by subcutaneous injection of 1 ml of sterile solution containing 0.2 mg ovalbumin and 200 mg $Al(OH)_3$ in saline; at the same time, 1% (w/v) of ovalbumin in saline was inhaled for 10 min (NE-U12, OMRON, Tokyo, Japan) (day 14). The animals were studied on the 21st day after the first sensitization (day 21).

2.4. Measurement of pulmonary mechanics

The animals were anesthetized intraperitoneally with 2.7 ml/kg of 33% (w/v) carbamic acid ethylester containing 0.83% (w/v) α-chloralose. The rats were tracheotomized by the insertion of a polyethylene cannula (inner diameter = 1.5 mm, Natsumeseisakusho, Tokyo, Japan) into the trachea to determine the intratracheal pressure and flow ($\dot{V}$). A polyethylene catheter (inner diameter

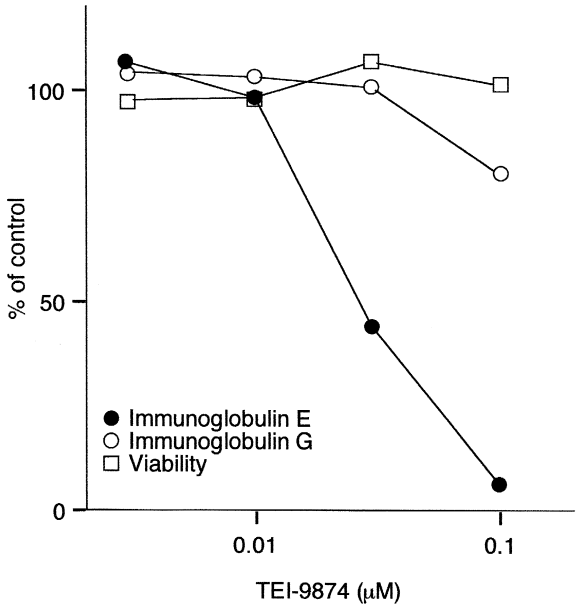

Fig. 1. Effect of TEI-9874 on the immunoglobulin production from human peripheral blood mononuclear cells in vitro. Human peripheral mononuclear cells were stimulated with the combination of human recombinant interleukin-4, 10, and mouse monoclonal anti-human CD40 antibody for 2 weeks. At the same time, cell viability was measured with alamar blue. Suppression (%) was calculated from the following equation: (level without TEI-9874 − level with TEI-9874)/level without TEI-9874 ×100. Results are expressed as the value for a single well.

= 1.2 mm, Natsumeseisakusho) was placed in the esophagus. Transpulmonary pressure (P_{tp}) was measured with a pressure transducer (Model DP-45-28; Medical Interface Project Station, Osaka, Japan) with one side attached to a saline-filled catheter inserted into the esophagus to determine the intraesophageal pressure and the other side attached to a catheter connected to a side port of the intratracheal cannula to determine the intratracheal pressure. Transpulmonary pressure was calculated as the difference between the intraesophageal pressure and the intratracheal pressure. Flow was measured with a pneumotachometer (Model HR8430B; Hans Rudolph, KS, USA), which was placed between the ventilator and the intratracheal cannula, connected to a different transducer (Model DP-45-14; Medical Interface Project Station). Transpulmonary pressure and flow signals were analyzed by using commercial PULMOS II software (Medical Interface Project Station). Lung resistance (R_L) was calculated with PULMOS II software by fitting the data to the following equation: $R_L = \Delta P_{tp}/\Delta \dot{V}$. The animals were then mechanically ventilated (Model 683; Harvard Apparatus, South Natick, MA, USA) at a frequency of 60 beats/min, a tidal volume of 10 ml/kg, and a positive end-expiratory pressure of 3-cmH_2O, and kept warm on a heating plate (HP-4530, Iuchi Eiseido, Osaka, Japan). The measurements of P_{tp} and $\dot{V}$ were made continously throughout the respiratory cycle.

2.5. Allergen provocation

The aerosolized saline and ovalbumin were generated by an ultrasonic nebulizer (NE-U06, OMRON) and delivered through the inspiratory line into the trachea. Measurements of 30-s duration were done during tidal ventilation before inhalation of the saline or ovalbumin aerosol. This represented the baseline measurement for each animal. Then, aerosolized saline or 2% (w/v) ovalbumin was inhaled for 5 min. In all animals, measurements of R_L were made at 2 min after the end of aerosol administration and repeated at 1-h intervals for up to 6 h. Each R_L is shown as % increase from baseline R_L in Section 3.

2.6. Airway hyperresponsiveness to acetyl-β-methylcholine chloride

All animals were sensitized as described in Section 2.3. On day 21, provocation with aerosolized 2% (w/v) ovalbumin was carried out for 25 min with the rats in the conscious state (NE-U12, OMRON). At 24 h after the ovalbumin provocation, airway hyperresponsiveness to acetyl-β-methylcholine chloride was measured with the animals anesthetized. The aerosols of doubling concentra-

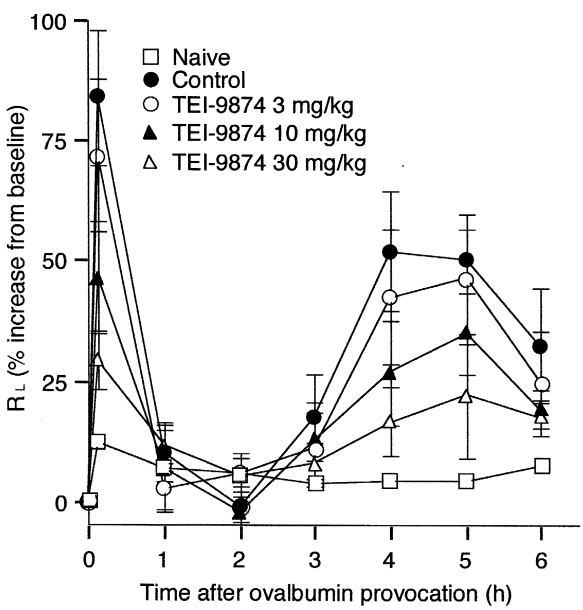

Fig. 2. Effect of TEI-9874 on ovalbumin-induced immediate-phase asthmatic response and late-phase asthmatic response in actively sensitized rats. The immediate-phase asthmatic response was an increase of R_L 2 min after the end of ovalbumin provocation. The late-phase asthmatic response was calculated as $AUC_{3-6\,h}$, which indicates the area under the curve of increase in R_L from 3 to 6 h after ovalbumin provocation. Naive group indicates non-sensitized and saline-provoked animals. TEI-9874 (3, 10, and 30 mg/kg) was administered orally for 5 days prior to ovalbumin provocation. Results are shown as the means ± S.E.M. Naive group consisted of two animals. Control and TEI-9874-treated groups consisted of seven or eight animals. TEI-9874 (30 mg/kg) reduced significantly both the immediate-phase asthmatic response and the late-phase asthmatic response compared with the control group value ($P < 0.05$).

Table 1
Effect of TEI-9874 on the numbers of leukocytes infiltrating into bronchoalveolar lavage fluid in actively sensitized rats after ovalbumin provocation

Treatment	*N*	Total	Neu	Eo	Mo
Naive	2	0.21	0.00	0.00	0.21
Control	8	10.44 ± 2.60	7.78 ± 2.03	1.04 ± 0.35	1.63 ± 0.26
TEI-9874 3 mg/kg	7	7.08 ± 1.39	4.71 ± 1.09	0.29 ± 0.08[a]	2.09 ± 0.56
TEI-9874 10 mg/kg	7	8.24 ± 2.17	5.87 ± 1.77	0.26 ± 0.08[a]	2.11 ± 0.38
TEI-9874 30 mg/kg	7	5.05 ± 1.11	3.18 ± 0.84	0.07 ± 0.02[b]	1.81 ± 0.30

Bronchoalveolar lavage was performed 6 h after inhalation of aerosolized ovalbumin. TEI-9874 (3, 10, 30 mg/kg) was given orally once a day for 5 days prior to ovalbumin provocation. All values ($\times 10^6$) represent the means ± S.E.M. Total: total leukocytes. Neu: neutrophils. Eo: eosinophils. Mo: mononuclear cells.

[a] $P < 0.05$.

[b] $P < 0.01$ compared with the control group value.

tions of acetyl-β-methylcholine chloride were generated with a nebulizer (NE-U06, OMRON) connected to the inspiratory line from the ventilator. The aerosols were administered intratracheally for 30 s at 5-min intervals, and the resulting bronchoconstriction was recorded as the % change of baseline of R_L. Baseline R_L was measured at 1 min after administration of aerosolized saline for 30 s. For each treatment group, the provoking concentration of aerosolized acetyl-β-methylcholine chloride causing a change in R_L of 200% (PC_{200}) was calculated by log–linear interpolation between the two concentrations bounding the point at which R_L reached 200% of baseline.

2.7. Bronchoalveolar lavage

Bronchoalveolar lavage was performed at the end of the R_L measurement, 6 h after ovalbumin provocation. The lungs were lavaged through the endotracheal tube with 8 ml of chilled saline. The total leukocyte number was counted by light microscopy with Turk solution. Cytospin slides were prepared using a cytospin (Auto Smear CF-12D, SAKURA, Tokyo, Japan) and air-dried for 5 min. Diff-Quick stain was used for the cellular differential count.

2.8. Enzyme-linked immunosorbent assay

IgE and IgG concentrations in the supernatants of human peripheral blood mononuclear cells cultures were measured by enzyme-linked immunosorbent assay (Nonoyama et al., 1993). Briefly, 96-well microtiter plates (Immulon 2; Dynatech Lab., Chantilly, VA) were coated with 50 μl of either goat anti-human IgG antibody or rabbit anti-human IgE antibody (2 μg/ml), and kept overnight at 4°C. After blocking of the wells with PBS containing 1% bovine serum albumin (200 μl), serially diluted test samples or standards (50 μl) were added to each well and incubated at room temperature for 2 h. After three washes with 300 μl of washing buffer containing 0.05% (w/v) Tween 20-PBS, secondary antibodies (50 μl), i.e. alkaline phosphatase-conjugated goat anti-human IgG antibody or biotin-conjugated goat anti-human IgE antibody were added, and incubation was continued for 2 h. For the measurement of IgE, alkaline phosphatase-conjugated extravidin was also used. Substrate solution containinig *p*-nitrophenyl phosphate disodium was prepared at a concentration of 1 mg/ml in carbonate buffer (pH 9.8) with 10 mM $MgCl_2 \cdot 6H_2O$. Optical density was determined with an automated enzyme-linked immunosorbent assay plate reader (Beckman Instuments, Fullerton, CA). The system was standardized with human IgE and IgG.

The enzyme-linked immunosorbent assay for the determination of rat immunoglobulins was carried out as follows: disposable sterile polystyrene enzyme-linked immunosorbent assay plates (Falcon, NJ) with 96 wells were coated with 50 μl of mouse monoclonal anti-rat IgE, G_1, or G_{2a} antibody (2 μg/ml) in PBS containing 0.05% (w/v) NaN_3 overnight at 4°C. The wells of the plate were washed three times with 300 μl of washing buffer containing 0.05% (w/v) Tween 20-PBS and then incubated overnight at 4°C with 200 μl of PBS containing both 5% (w/v) bovine serum albumin and 0.05% (w/v) NaN_3 in PBS as the blocking step. Test serum (50 μl) serially diluted with dilution buffer containing both 1% (w/v) bovine serum albumin and 0.05% (w/v) NaN_3 was added to each well, and the plate was then incubated for 1 h at room temperature. Following a wash with 300 μl of

Table 2
Effect of TEI-9874 on serum anti-ovalbumin immunoglobulin levels in actively sensitized rats

Treatment	*N*	Anti-ovalbumin IgE	Anti-ovalbumin IgG_1	Anti-ovalbumin IgG_{2a}
Naive	2	0.13	0.65	0.32
Control	8	1.07 ± 0.24	15,753 ± 2556	8003 ± 1893
TEI-9874 3 mg/kg	7	0.97 ± 0.25	12,387 ± 906	6352 ± 510
TEI-9874 10 mg/kg	7	0.43 ± 0.07[a]	10,398 ± 791	5699 ± 580
TEI-9874 30 mg/kg	7	0.31 ± 0.05[b]	7009 ± 1008[b]	3944 ± 854

TEI-9874 (3, 10, 30 mg/kg) was given orally once a day for 5 days prior to ovalbumin provocation. All values (titer) represent the means ± S.E.M.

[a] $P < 0.05$.

[b] $P < 0.01$ compared with the control group value.

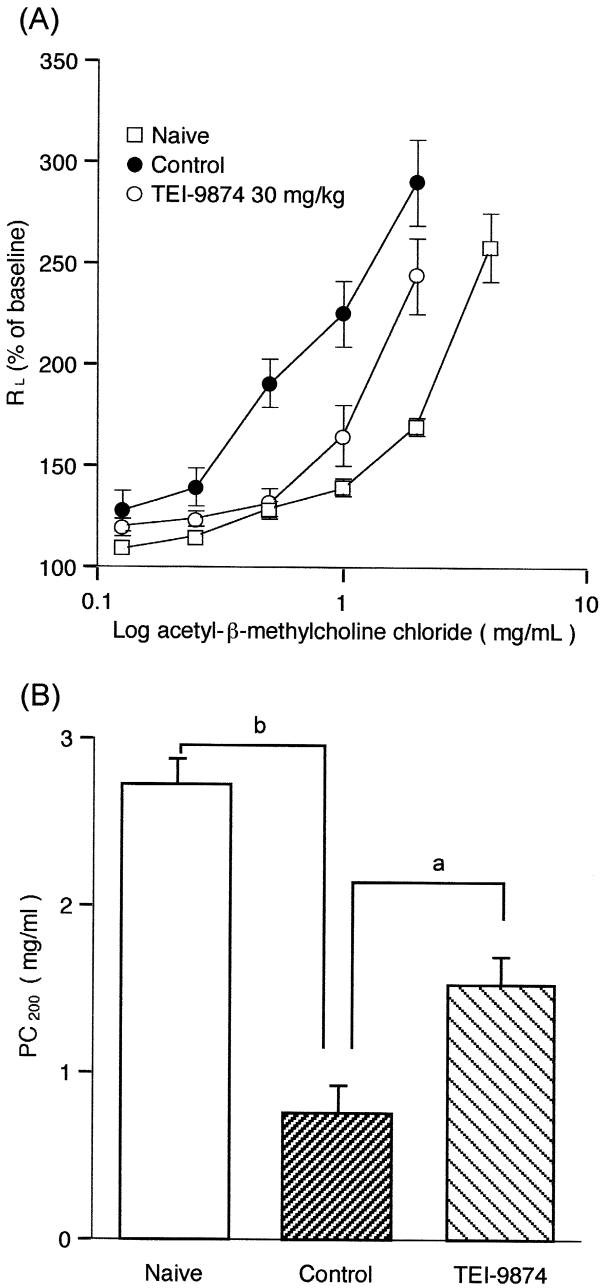

Fig. 3. Effect of TEI-9874 on ovalbumin-induced airway hyperresponsiveness curve (A) and PC_{200} (B) to acetyl-β-methylcholine chloride in actively sensitized rats. PC_{200} indicates the provoking concentration of aerosolized acetyl-β-methylcholine chloride causing a change in R_L of 200%, which was calculated by logarithmic regression analysis. TEI-9874 (30 mg/kg) was given orally for 5 continuous days prior to ovalbumin provocation. Open square indicates naive (non-sensitized, non-provoked) animals. Results are shown as the means ± S.E.M. for eight animals. $^{a}P < 0.01$, $^{b}P < 0.001$ compared with naive or control group value.

washing buffer, the wells were incubated for 1 h at room temperature with 50 μl of biotin-labeled ovalbumin (1 μg/ml) in dilution buffer. After washing, the plate was incubated for 0.5 h at room temperature with 50 μl of avidin–biotin–horseradish peroxidase and was then washed. 3,3′5,5′-Tetramethylbenzidine microwell peroxidase substrate solution (100 μl) was added to the wells, and the plate was incubated for 15 min at room temperature; and then the reaction was stopped by the addition of 2 N H_2SO_4 (100 μl). Spectrophotometric readings were then made using 450-nm (main), 540-nm (reference) wavelength filters of a dual wavelength microplate reader (Molecular Devices, Germany). A standard curve for the determination of immunoglobulins in rat serum was prepared with serum from a rat sensitized with ovalbumin. Results were expressed as enzyme-linked immunosorbent assay titers using the midpoint of the titration curves obtained as compared with a constant standard run in each assay.

2.9. Rat anti-ovalbumin antiserum

Brown–Norway rats (140–190 g), 8 weeks of age, were used. Sensitization was performed in the same manner as used for the rat biphasic asthmatic model. Rat anti-ovalbumin antiserum was obtained at day 17. Anti-ovalbumin IgE, G_1, and G_{2a} titers estimated by enzyme-linked immunosorbent assay in anti-ovalbumin antiserum were 2.6, 9055, and 3349, respectively. Naive serum was obtained from non-sensitized naive rats, 10 weeks of age. Anti-ovalbumin IgE, G_1, and G_{2a} titers estimated by enzyme-linked immunosorbent assay in naive serum were 0.12, 0.58, and 0.38, respectively.

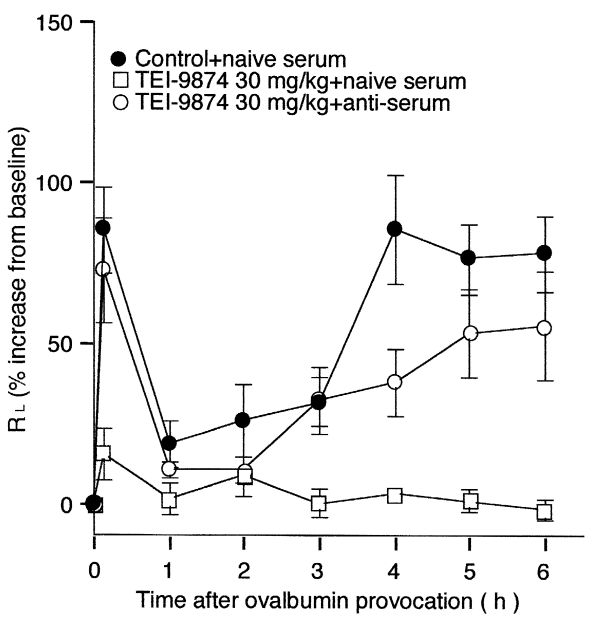

Fig. 4. Reversal effect of exogenous rat anti-ovalbumin antiserum on the reduction of the immediate-phase asthmatic response and the late-phase asthmatic response in actively sensitized rats, pretreated with TEI-9874. TEI-9874 (30 mg/kg) was given orally for 5 days prior to ovalbumin provocation. Rat anti-ovalbumin antiserum or naive serum was administered intravenously at days 16 and 20. Results are shown as the means ± S.E.M. for six or seven animals.

Table 3
Effect of TEI-9874 on serum anti-ovalbumin immunoglobulin levels in actively sensitized rats

Treatment	*N*	Anti-ovalbumin IgE	Anti-ovalbumin IgG_1	Anti-ovalbumin IgG_{2a}
Naive	2	0.17	0.63	0.44
Control + naive serum	6	0.96 ± 0.20	18,220 ± 2484	6268 ± 951
TEI-9874 30 mg/kg + naive serum	7	0.18 ± 0.02 (98.7)	11,567 ± 1506 (36.5)	5435 ± 616 (13.3)

TEI-9874 (30 mg/kg) was given orally once a day for 5 days prior to ovalbumin provocation. All values (titer) represent the means ± S.E.M. Values in parentheses represent percent inhibition.

2.10. Protocol

The study was divided into four separate protocols. In the first protocol, the rats were treated once a day for 5 days (from day 16 to day 20 following the first sensitization) with TEI-9874 (3, 10, or 30 mg/kg p.o.) prior to the ovalbumin provocation. In the second protocol, to determine airway hyperresponsiveness to acetyl-β-methylcholine chloride, the rats were treated once a day for 5 days with TEI-9874 (30 mg/kg p.o.) prior to the ovalbumin provocation. In the third protocol, we examined whether the reduction of biphasic airway response by the treatment with TEI-9874 might be due to suppression of the production of anti-ovalbumin IgE or not. That is to say, rat anti-ovalbumin antiserum (2 ml/animal), in which anti-ovalbumin IgE was rich (2.6 titer), or naive rat serum (2 ml/animal) was transferred twice (day 16 and day 20 i.v.) into the actively sensitized rats that were treated once a day for 5 days with TEI-9874 (30 mg/kg p.o.) prior to the ovalbumin provocation. In addition, naive serum was transfered into control animals. In the last protocol, the rats were treated 2 h with TEI-9874 (30 mg/kg p.o.) prior to the ovalbumin provocation. All experiments were carried out in accordance with "Standards for Animal Care and Use" of the "Animal Experiment Ethics Committee of the Teijin Institute for Bio-Medical Research, Teijin".

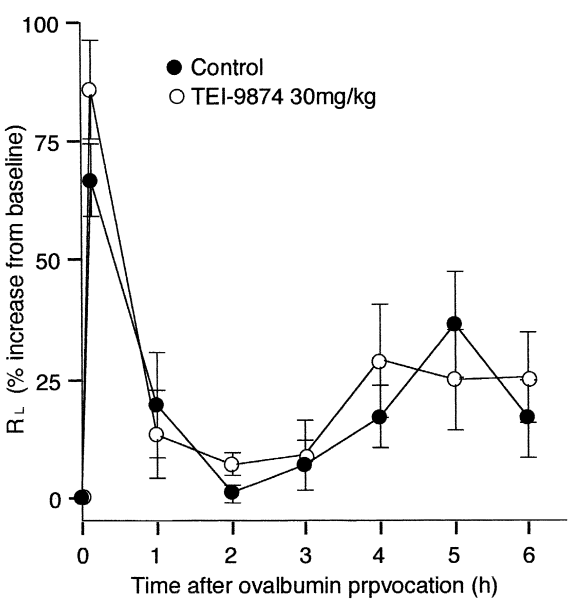

Fig. 5. Effect of single treatment with TEI-9874 on ovalbumin-induced immediate-phase asthmatic response and late-phase asthmatic response in actively sensitized rats. The immediate-phase asthmatic response was an increase of R_L 2 min after the end of ovalbumin provocation. The late-phase asthmatic response was calculated as $AUC_{3-6\ h}$, which indicates the area under the curve for the increase in R_L from 3 to 6 h after the ovalbumin provocation. TEI-9874 (30 mg/kg) was administered orally 2 h prior to ovalbumin provocation. Results are shown as the means ± S.E.M. for six animals.

2.11. Statistical analysis

The data were expressed as the means ± S.E.M. Groups were compared by Student's standard two-tailed *t*-test and by Dunnett's multiple comparison test when appropriate. Groups were considered different if the *P* value was less than 0.05.

3. Results

3.1. Effect of TEI-9874 on immunoglobulin production from human peripheral blood mononuclear cells in vitro

Peripheral blood mononuclear cells from a normal volunteer produced IgE and IgG on treatment with the combination of human recombinant interleukin-4, 10, and mouse monoclonal anti-human CD40 antibody. TEI-9874 demonstrated strong suppression of IgE production with an IC_{50} of 27 nM, in comparison with its efficacy for IgG with an IC_{25} of more than 100 nM. In this case, the viability of cells was more than 98% (Fig. 1).

Table 4
Effect of single administration of TEI-9874 on the numbers of leukocytes infiltrating into bronchoalveolar lavage fluid in actively sensitized rats

Treatment	*N*	Total	Neu	Eo	Mo
Naive	2	0.74	0.09	0.04	0.60
Control	6	3.33 ± 1.03	2.58 ± 0.95	0.20 ± 0.05	0.55 ± 0.08
TEI-9874 30 mg/kg	6	3.45 ± 0.52	2.58 ± 0.51	0.28 ± 0.03	0.59 ± 0.09

Bronchoalveolar lavage was performed 6 h after inhalation of aerosolized ovalbumin. TEI-9874 (30 mg/kg) was given orally 2 h prior to the ovalbumin provocation. All values ($\times 10^6$) represent the means ± S.E.M. Total: total leukocytes. Neu: neutrophils. Eo: eosinophils. Mo: mononuclear cells.

Table 5
Effect of single administration of TEI-9874 on serum anti-ovalbumin immunoglobulin levels in actively sensitized rats

Treatment	*N*	Anti-ovalbumin IgE	Anti-ovalbumin IgG_1	Anti-ovalbumin IgG_{2a}
Naive	2	0.06	0.45	0.38
Control	6	0.72 ± 0.09	22,173 ± 3496	7776 ± 1503
TEI-9874 30 mg/kg	6	1.14 ± 0.20	28,188 ± 1233	15,168 ± 2580

TEI-9874 (30 mg/kg) was given orally 2 h prior to the ovalbumin provocation. All values (titer) represent the means ± S.E.M.

3.2. Effect of TEI-9874 on ovalbumin-induced immediate-phase asthmatic response and late-phase asthmatic response in anesthetized rats

To make an allergen-induced biphasic asthmatic model, we tried sensitization with ovalbumin, $Al(OH)_3$, and inactivated *B. pertussiss* vaccine in Brown–Norway rats. On day 21, the baseline measurement of R_L was 230.1 ± 4.1 $cmH_2O\ l^{-1}\ s^{-1}$. The provocation with aerosolized ovalbumin produced a biphasic asthmatic response consisting of the immediate-phase asthmatic response and the late-phase asthmatic response that occurred 3–6 h after ovalbumin provocation in actively sensitized rats (Fig. 2).

In the first study, both the immediate-phase asthmatic response and the late-phase asthmatic response were reduced by the treatment with TEI-9874 (3, 10, and 30 mg/kg p.o.) for 5 days prior to the ovalbumin provocation, with an ED_{50} of 10.3 and 13.5 mg/kg, respectively, in a dose-dependent manner (Fig. 2). In this case, the number of eosinophils that infiltrated into the bronchoalveolar lavage fluid was reduced by 72.1%, 75.0%, and 93.3%, respectively. Furthermore, the numbers of total leukocytes and neutrophils were reduced (Table 1). Also, the serum anti-ovalbumin IgE level was suppressed by 10.6%, 68.1%, and 80.9%, respectively, with an ED_{50} of 8.7 mg/kg, although other immunoglobulin levels were only decreased slightly (Table 2).

3.3. Effect of TEI-9874 on the airway hyperresponsiveness to inhaled acetyl-β-methylcholine chloride

In the second study, aerosolized acetyl-β-methylcholine chloride caused a dose-dependent bronchoconstriction in naive (unsensitized, unprovoked) animals (PC_{200} = 2.72 mg/ml). In sensitized, provoked animals, the dose–response curve to acetyl-β-methylcholine chloride was shifted to the left (PC_{200} = 0.76 mg/ml), and there was a significant difference in airway hyperresponsiveness (measured as % change of baseline R_L) from naive animals ($P < 0.001$). In sensitized, provoked animals that had received TEI-9874, the reactivity to acetyl-β-methylcholine chloride was reduced significantly (PC_{200} = 1.52 mg/ml, $P < 0.01$) compared with that of sensitized, provoked animals (Fig. 3A,B).

3.4. Reversal effect of the exogenous administration of anti-ovalbumin IgE on the reduction of the immediate-phase asthmatic response and the late-phase asthmatic response by the pre-treatment with TEI-9874

In the third study, the treatment with TEI-9874 (30 mg/kg p.o.) for 5 days prior to the ovalbumin provocation reduced the biphasic asthmatic responses (Fig. 4). In this case, TEI-9874 completely reduced the increase of serum anti-ovalbumin IgE by 98.7%, although the increases of anti-ovalbumin IgG_1, anti-ovalbumin IgG_{2a} were reduced slightly, by 36.5% and 13.3%, respectively (Table 3). Under these conditions, the suppression of the immediate-phase asthmatic response by the treatment with TEI-9874 was almost completely extinguished by the additional injection of the rat anti-ovalbumin antiserum, which was rich in anti-ovalbumin IgE (2.6 titer). The inhibitory effect of TEI-9874 on the late-phase asthmatic response was also mostly extinguished by the injection of this antiserum (Fig. 4). On the other hand, naive rat serum had no reversal effect on the suppression of the immediate-phase asthmatic response and late-phase asthmatic response by the treatment with TEI-9874.

3.5. Effect of single administration of TEI-9874 on ovalbumin-induced immediate-phase asthmatic response and late-phase asthmatic response in anesthetized rats

In the last study, the immediate-phase asthmatic response, the late-phase asthmatic response, and inflammatory cell numbers in the bronchoalveolar lavage fluid were not reduced by the single treatment with TEI-9874 2 h before the ovalbumin provocation (Fig. 5, Table 4). The serum anti-ovalbumin IgE, IgG_1, and IgG_{2a} levels were also not reduced (Table 5).

4. Discussion

IgE is thought to be an important mediator in the pathogenesis of allergic disorders, including allergic asthma (Richard, 1993; Coyle et al., 1996). It seems that the targeting of IgE in allergic disorders may follow two strategies. One is the neutralization of allergen specific IgE by anti-IgE antibody (Coyle et al., 1996) or the soluble

α-chain of FcεRI (Naito et al., 1995, 1996). The other strategy may be the inhibition of immunoglobulin production in B lymphocytes. The first therapeutic strategy demonstrated therapeutic efficacy in asthmatic subjects (John et al., 1997; Boulet et al., 1997). In the current study with actively sensitized Brown–Norway rats, we examined whether the second strategy might be an effective treatment for allergic asthma.

In allergic asthmatic patients, the allergen-specific IgE level is chronically maintained at a high level, and bronchial mast cells are already sensitized with allergen-specific IgE. Likewise, we speculate that mast cells were already sensitized with anti-ovalbumin IgE at the second sensitization in our animal model, because the serum anti-ovalbumin IgE was somewhat elevated (0.5 titer). In this case, TEI-9874 reduced both the immediate-phase asthmatic response and the late-phase asthmatic response in a dose-dependent manner (Fig. 2) even when treatment started 2 days after the second sensitization. Also, the increase in anti-ovalbumin IgE level was greately reduced by the treatment with TEI-9874 (Table 2). This result indicates that treatment with TEI-9874 might be of significant use in the therapy for chronic asthma in which bronchial mast cells have been already sensitized with allergen-specific IgE.

It has been reported that IgE causes the immediate-phase asthmatic response by the activation of bronchial mast cells (Peters, 1990; Schulman, 1993; Richard, 1993), but the role of IgE in the late-phase asthmatic response is less certain. Our study aimed to clarify whether the treatment with TEI-9874 might exert its inhibitory effect on the late-phase asthmatic response via suppression of anti-ovalbumin IgE production. That is to say, rat anti-ovalbumin antiserum was transferred twice (days 16 and 20) into the actively sensitized rats whose anti-ovalbumin IgE synthesis was completely suppressed by the pretreatment with TEI-9874. The rat anti-ovalbumin antiserum used in our study exhibited a very high level (2.6 titer) of ovalbumin-specific IgE, which was estimated by enzyme-linked immunosorbent assay. In this case, the combination of TEI-9874 and naive serum almost completely reduced both biphasic asthmatic response and the increase in serum anti-ovalbumin IgE (by 98.7%), although anti-ovalbumin IgG_1 and IgG_{2a} were slightly reduced, by 36.5% and 13.3%, respectively. Under these conditions, the suppression of the immediate-phase asthmatic response and the late-phase asthmatic response by the treatment with TEI-9874 was completely eliminated by the additional injection of exogenous rat anti-ovalbumin antiserum, in which anti-ovalbumin IgE abounded, instead of naive serum (Fig. 4). It seems that, in this study, exogenous rat antiserum-derived anti-ovalbumin IgGs, which binds to bronchial mast cells, would already have been cleared from the mast cell surface at the ovalbumin provocation, and the last injection of exogenous rat anti-ovalbumin antiserum was carried out 24 h before the ovalbumin provocation. Therefore, it is unlikely that anti-ovalbumin IgGs participated in this reversal of the inhibition of the immediate-phase asthmatic response and the late-phase asthmatic response induced by the additional treatment with rat anti-ovalbumin antiserum. Moreover, a single treatment with TEI-9874 (30 mg/kg p.o.), given 2 h before the ovalbumin provocation, had no effect on ovalbumin-induced immediate-phase asthmatic response, late-phase asthmatic response, the infiltration of inflammatory cells into respiratory tract, or the anti-ovalbumin IgE level (Fig. 5, Tables 4 and 5). Based on these various resuts, it seems that TEI-9874 might inhibit mainly the allergen-induced immediate-phase asthmatic response and the late-phase asthmatic response via suppression of anti-ovalbumin IgE production.

There are a few possible explanations for the fact that the inhibitory effect of TEI-9874 on the late-phase asthmatic response was not totally extinguished by the rat anti-ovalbumin antiserum, as there remained 38% inhibition. The amount of rat anti-ovalbumin antiserum used in our study might not have been sufficient to reverse the inhibition of the late-phase asthmatic response induced by the treatment with TEI-9874. However, this is probably unlikely, because the suppression of the immediate-phase asthmatic response was completely extinguished. On the other hand, activated T cells have been implicated in the late-phase asthmatic response. However, the effect of TEI-9874 on T cell function still remains to be examined.

Finally, TEI-9874 reduced effectively both the infiltration of inflammatory cells into bronchoalveolar lavage fluid and the airway hyperresponsiveness to acetyl-β-methylcholine chloride stimulation. Migration of these cells has been thought to be induced by leukotriene B4, platelet activating factor, and interleukin-5, all of which are produced by activated mast cells. TEI-9874 might reduce the release of these chemoattractants from mast cells, not via a direct effect on mast cells, but via the inhibition of anti-ovalbumin IgE production, because we confirmed that single treatment with TEI-9874 did not have any effect on the immediate-phase asthmatic response, the late-phase asthmatic response, or inflammatory cell migration. Mast cells provide inflammatory mediators, including histamine, leukotrienes, neutral proteases, and chemoattractants, which cause bronchoconstriction, increased bronchovascular permeability, and bronchial eosinophilia (Holgate et al., 1988; Peters et al., 1989; Sommerhoff et al., 1989; Evans et al., 1989; Peters, 1990; Tokuyama et al., 1991). Moreover, migrated eosinophils injure bronchial epithelial cells via their toxic peptides (Gleich et al., 1979; Ayars et al., 1985; Hastie et al., 1987; Motojima et al., 1989; Underwood et al., 1995). These inflammatory factors cause the late-phase asthmatic response and lead to the airway hyperresponsiveness. Therefore, it seems that TEI-9874 might express its suppressive effects on the late-phase asthmatic response and airway hyperresponsiveness by inhibiting the release from the mast cell of these mediators that produce bronchial damage.

In conclusion, treatment of a rat allergic asthmatic model with TEI-9874 significantly reduced serum allergen-specific IgE concentrations and attenuated the immediate-phase asthmatic response, the late-phase asthmatic response, and respiratory leukocytosis to allergen provocation. Our study establishes the involvement of IgE in the pathogenesis of both the immediate-phase asthmatic response and the late-phase asthmatic response to allergen provocation, and suggests that TEI-9874 may be a good candidate for the therapy of allergic disorders due to allergen-specific IgE, including allergic asthma.

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