

Inhibition of Airway Inflammation in rDer f 2-Sensitized Mice by Oral Administration of Recombinant Der f 2

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Recombinant Der f 2 (rDer f 2) is a promising new allergen expected to improve the diagnosis and immunotherapy of house dust mite allergy and to further immunological studies. To evaluate the hyposensitizing activity of rDer f 2 to mite allergy, we examined the effect of its oral administration on allergic inflammation in A/J mice immunized with mite allergens. A/J mice immunized with rDer f 2 alone or rDer f 2 + crude mite extract were orally given 0 (control), 0.01, 0.1, or 1 mg/day of rDer f 2 for 4 weeks, followed by an antigen inhalation challenge. Twenty-four hours after rDer f 2 inhalation, control animals experienced severe leukocyte influx into the airway. The infiltrating cells were mainly neutrophils, with some eosinophils and lymphocytes. The concentrations of IL4, IFN γ , and soluble ICAM-1 in the bronchial alveolar lavage fluid increased twofold compared with values before rDer f 2 inhalation. In contrast, inflammation was significantly suppressed in mice given 1 mg/day of rDer f 2 orally for 4 weeks and partially suppressed in those fed 0.1 mg/day of the antigen. Plasma anti-rDer f 2 antibody levels were unchanged by oral rDer f 2 treatment. Mite extract inhalation challenge provoked neutrophilia in rDer f 2 + mite-sensitized control mice, and this allergic reaction tended to decrease in sensitized mice fed 1 mg/day of rDer f 2 orally for 4 weeks. We conclude that rDer f 2 has hyposensitizing activities and may be useful in immunotherapy for house dust mite allergy. © 1997 Academic Press

INTRODUCTION

To date, several clinically important allergens have been identified in body and culture extracts of the house dust mite, *Dermatophagoides farinae* (1, 2). Among these, Der f 2 has had its structural gene cloned, and the product of this cloned gene (rDer f 2) was shown to exhibit allergic activity in humans comparable with native Der f 2 prepared from mite extract (3). There-

fore, rDer f 2 is potentially useful in allergic immunotherapy.

Evidence supports that the late-phase asthmatic reaction (LAR), but not the immediate reaction, closely resembles the natural history and severity of certain allergic diseases (4–6). LAR occurs 6–48 hr after allergen challenge and is always accompanied by severe infiltration of leukocytes into airway tissue. Such reactions are long lasting and sometimes difficult to manage. While administration of nonbronchodilator or prophylactic drugs is commonly used in the treatment of LAR, immunotherapy with sensitizing allergens has also been tried (5–7).

In the present study, we examined the effect of oral rDer f 2 administration on antigen-induced airway inflammation characterized by leukocyte influx in a mite-sensitized A/J mice strain highly responsive to mite allergens.

MATERIALS AND METHODS

We performed two experiments. In experiment 1, mice were immunized and challenged with rDer f 2 alone. In experiment 2, mice were cosensitized with rDer f 2 and crude mite extract and challenged with crude mite extract. Animals were fed PBS or rDer f 2 solution in both experiments (Fig. 1).

Antigen

Recombinant Der f 2, clone 1 (3), was kindly supplied by the Nikka Whisky Distilling Co., Ltd. (Chiba, Japan). Crude body extract from house dust mite, *D. farinae* (mite extract), was kindly supplied by Torii & Co., Ltd. (Chiba, Japan). This mite extract contained about 0.3% (w/w) of native Der f 2, as determined by enzyme-linked immunosorbent assay. Allergens were dissolved in PBS before use.

Animals

Male A/J mice, 6 weeks of age, were purchased from Japan SLC, Inc. (Shizuoka, Japan).

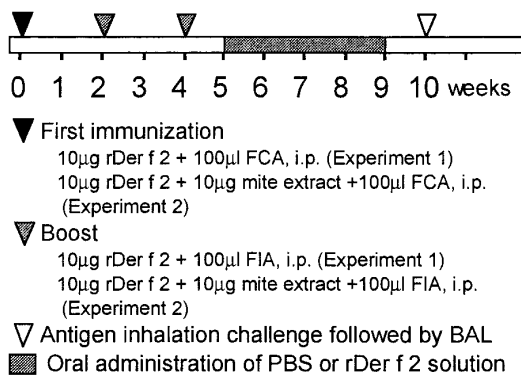


FIG. 1. Experimental schedule. rDer f 2-sensitized A/J mice were challenged with rDer f 2 in experiment 1. Mice coimmunized with rDer f 2 and mite extract were challenged with mite extract in experiment 2. Animals were fed PBS or rDer f 2 in both experiments during the administration period.

Experiment 1

Immunization. Allergen solution containing 10 μ g of rDer f 2 and 100 μ l of Freund's complete adjuvant (FCA) was injected intraperitoneally into 7-week-old A/J mice as the first immunization. After 14 and 28 days, 10 μ g of rDer f 2 and 100 μ l of Freund's incomplete adjuvant (FIA) were given intraperitoneally as a booster.

Several mice without immunization ("naive mice") were also used as reaction-negative controls.

Experimental groups and oral administration of rDer f 2. rDer f 2-sensitized mice were divided into three rDer f 2-fed groups (0.01, 0.1, and 1 mg/day rDer f 2) and a PBS-fed group (as control). Each group consisted of 7–10 animals. Oral administration was started 7 days after the last immunization. Animals were given 0.25 ml of rDer f 2 solution or PBS 5 sequential days a week for 4 weeks using a syringe fitted with an 18-gauge stainless steel needle. Mice in the 3 rDer f 2-fed groups received 0.01, 0.1, or 1 mg/body wt/day of rDer f 2.

Naive mice received no treatment over the experimental period.

Induction of allergic airway inflammation. An antigen inhalation challenge followed by bronchial alveolar lavage was carried out 3 days after the last oral administration. Mice were challenged with rDer f 2. Leukocyte numbers and biochemical activities in the bronchial alveolar lavage fluid (BALF) were determined as an index for allergic airway inflammation.

An aerosol mist of 10 mg/ml rDer f 2 solution was generated by an ultrasonic nebulizer and inhaled by a mouse confined in a small chamber for 30 min. After 6 or 24 hr, the animals were anesthetized by an excess amount of pentobarbital sodium (100 mg/kg, ip) and killed by bleeding. Tracheal cannulation was then per-

formed with a PE-50 polyethylene tube. PBS (0.7 ml) was injected into the lung through the tube, followed by the recovery of BALF. This procedure was repeated once more to yield 1.0–1.2 ml of BALF from each mouse. After counting the total cell number in the BALF, cytospin preparations were made from 0.2-ml aliquots of the BALF. The numbers of macrophages, neutrophils, eosinophils, and lymphocytes were determined by differential leukocyte counting after Diff-quick stain, in which we examined about 300 cells/preparation. A part of the BALF was centrifuged at 1500 rpm for 15 min, and its supernatant was examined for "biochemical values," that is, total protein content, lactate dehydrogenase (LDH), and β -glucuronidase (GLU) activities and IL4, IL6, TNF α , IFN γ , and soluble ICAM-1 (sICAM-1) concentrations. Total protein, LDH, and GLU were determined by using an automatic enzyme analyzer (Fuji Dry-Chem, Fuji Photo Film Co., Ltd., Japan). Cytokines and sICAM-1 were measured by enzyme linked immunosorbent assays (ELISAs). It has been shown that total protein concentration and LDH and GLU activities in the BALF increase when airway is inflamed by physical/chemical damage (8, 9) and infection (10–12). Increased levels of proteins and LDH are mainly derived from damaged epithelia and leaked plasma (13, 14). GLU is released by activated neutrophils and macrophages (11, 15). Increases in cytokine and sICAM-1 concentrations in airway lavage fluids during allergic inflammations have also been reported (16–19). sICAM-1 are shed from the cell surface over expressing ICAM-1 molecules after activation (16, 20).

Determination of cytokine and sICAM-1 concentrations in BALFs. Cytokines and sICAM-1 levels were determined by ELISAs after recommendations from manufacturers of antibodies. We used following combinations of capturing and detecting antibodies: rat monoclonal anti-mouse IL4 (PM-18034A, PharMingen, San Diego, CA) and biotinylated rat monoclonal anti-mouse IL4 (PM-18042D, PharMingen); rat monoclonal anti-mouse IL6 (PM-18071D, PharMingen) and biotinylated rat monoclonal anti-mouse IL6 (PM-18082D, PharMingen); rat monoclonal anti-mouse IFN γ (PM-18181D, PharMingen) and biotinylated rat monoclonal anti-mouse IFN γ (PM-18112D, PharMingen); rat monoclonal anti-mouse TNF α (PM-18131D, PharMingen) and biotinylated rabbit polyclonal anti-mouse TNF α (PM-18352D, PharMingen); rat monoclonal anti-mouse ICAM-1 (270650, Seikagaku Corporation, Tokyo, Japan) and hamster polyclonal anti-mouse ICAM-1 (PM-01541D, PharMingen). Detecting antibodies were labeled by avidin-conjugated β -galactosidase (A-2300, Vector, Burlingame, CA) or peroxidase-conjugated goat anti-hamster IgG (14-22-06, Kirkegaard & Perry Labora-

tories Inc., MD) for fluorescence or color development. Standard curves for determining sICAM-1 levels were obtained from the BALF at 24 hr after rDer f 2 inhalation of the PBS-fed group. Standard curves for cytokines were made by using recombinant mouse IL4 (PM-19231W, PharMingen), IL6 (PM-19251V, PharMingen), IFN γ (PM-19301U, PharMingen), and TNF α (PM-19321T, PharMingen).

Determination of plasma anti-rDer f 2 IgG levels. Heparinized blood was collected from the retroorbital plexus of individual mice on the day before the beginning of oral feeding and the day after the end of feeding in experiment 1. Plasma (about 20 μ l/animal) was separated from the whole blood and frozen until we measured IgG. Anti-rDer f 2 IgG1 and IgG2a were determined by ELISAs using rDer f 2 as a capturing antigen and two detecting antibodies, biotinylated rat monoclonal anti-mouse IgG1 (PM-05002D, PharMingen) and anti-mouse IgG2a (PM-05022D, PharMingen) as described elsewhere (3). Standard curves for determining IgG levels were obtained from the pooled plasma of the PBS-fed group collected before oral administration. The IgG levels of this plasma were taken as "1.0."

Statistical analysis. All the values were expressed as means with standard error. Statistical significance of difference was determined by Student's *t* test. A *P* value lower than 0.05 was considered significant.

Experiment 2

Except for the allergen used and several points mentioned below, the same procedures as in experiment 1 were employed.

Immunization. Allergen solution containing 10 μ g of mite extract, 10 μ g of rDer f 2, and 100 μ l of FCA was injected intraperitoneally into 7-week-old A/J mice as the first immunization. After 14 and 28 days, 10 μ g of rDer f 2 and 100 μ l of FIA were given intraperitoneally as a booster.

Experimental groups and oral administration of rDer f 2. Mice coimmunized with rDer f 2 and mite extract were divided into two rDer f 2-fed groups (0.1 and 1 mg/day rDer f 2) and a PBS-fed group (as control). Animals were fed PBS or rDer f 2 solutions for 4 weeks. Mice in the two rDer f 2-fed groups received 0.1 or 1 mg/body wt/day of rDer f 2.

Induction of allergic airway inflammation. Aerosol mist was generated from 10 mg/ml of crude mite extract and inhaled by mice for 30 min. Leukocyte numbers in the BALF were determined 24 hr after the inhalation challenge. Biochemical activities were not determined in experiment 2.

RESULTS

Side Effects Induced by rDer f 2 Administration

No anaphylactic response was observed in mice given rDer f 2 orally over the experimental period in either experiment 1 or experiment 2.

Time Course of Airway Inflammation after Antigen Inhalation

In a preliminary examination, we determined the time course of the airway inflammation in mice sensitized with rDer f 2 5 min (as 0 hr), 3, 6, 12, 24, and 48 hr after an inhalation of 10 mg/ml of rDer f 2 for 30 min. BALFs were prepared from four to six mice for each time point, leukocytes were counted, and cytokine and sICAM-1 concentrations were measured. Total leukocyte numbers in the BALF began to increase within 3 hr and maximized at 24–28 hr (two- to threefold increase compared to the base value at 0 hr), showing characteristics of late-phase inflammation (Fig. 2). The increasing cells were mainly neutrophils, with some eosinophils and lymphocytes. No such leukocyte influx into the airway was observed in the naive mice challenged with rDer f 2 or the sensitized mice subject to PBS or ovalbumin inhalation. The results suggested that the reaction was antigen specific. We also traced the changes in IL4, IL6, IFN γ , TNF α , and sICAM-1 levels of the BALFs after rDer f 2 challenge in sensitized mice. Each cytokine concentration had a different rise-and-fall pattern. TNF α and IL6 levels, known to be produced by several cell types including mast cells and macrophages, rose and fell rapidly. T-cell-specific cytokine, IL4, and IFN γ levels increased relatively late. sICAM-1 levels began to increase within 3 hr after allergen inhalation and remained high for over 48 hr with a time course that resembled the neutrophil count.

Effect of rDer f 2 Feeding on Allergic Inflammation in rDer f 2-Sensitized Mice in Experiment 1

The BALF of rDer f 2-sensitized mice fed PBS or rDer f 2 solutions was examined 6 or 24 hr after rDer f 2 inhalation in experiment 1. We chose the 6th hour for determining TNF α and IL6, and the 24th hour for examining overall inflammation, IL4, and IFN γ . In the PBS-fed group, total leukocyte numbers in the BALF were increased about twofold at 6 hr and about threefold at 24 hr after the challenge in comparison with naive mice (Fig. 3a). The protein content, the LDH and GLU activities (Fig. 3b), and the IL4, IFN γ , and sICAM-1 (Fig. 3c) levels in the BALF collected from PBS-fed animals 24 hr after inhalation also showed two- to threefold increases compared with naive mice. TNF α and IL6 levels 6 hr postchallenge in PBS-fed mice were 25–50% higher than that of naive mice (Fig. 3c). In

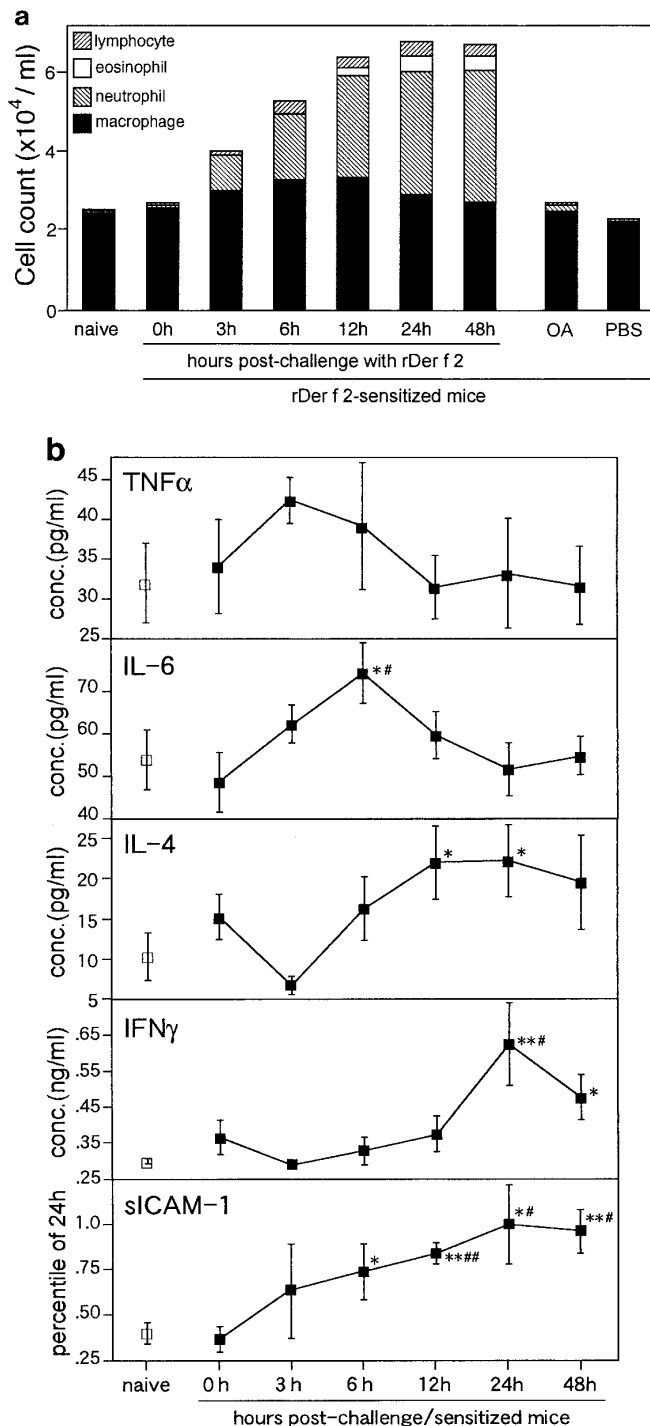


FIG. 2. (a) Time course of the infiltration by leukocytes into the airway after rDer f 2 inhalation for 30 min in rDer f 2-sensitized mice ($n = 4-6$ for each time point). Naive mice ($n = 5$) were examined 24 hr after the rDer f 2 inhalation challenge. Cell counts in sensitized mice challenged with PBS ($n = 4$) and ovalbumin ($n = 4$) are shown as "PBS" and "OA," respectively. Values were determined 24 hr after challenges. (b) Time course of IL4, IL6, TNF α , IFN γ , and soluble ICAM-1 in the BALF. BALF was obtained from the same animals as in (a). ** $P < 0.01$, * $P < 0.05$ compared with naive. *** $P < 0.01$, # $P < 0.05$ compared with 0 hr of sensitized animals.

contrast, these indexes of inflammation at 24 hr after rDer f 2 inhalation were suppressed in rDer f 2-fed groups in a dose-dependent manner compared to the PBS-fed group (Figs. 3a–3c). A significant difference was obtained in neutrophil count, protein content, LDH activities, IFN γ concentration, and sICAM-1 concentration between the 1 mg/day rDer f 2-fed group and the PBS-fed control group. Biochemical values and neutrophil numbers in sensitized animals correlated closely as shown in Table 1. Neutrophil count and TNF α and IL6 levels 6 hr postchallenge in the 1 mg/day rDer f 2 group were also lower than that of control. The difference was not significant, however.

Changes in Plasma IgG Titer in Experiment 1

Table 2 shows plasma anti-rDer f 2 IgG levels before and after oral administration in experiment 1. Anti-rDer f 2 IgGs were not detected in naive mice. rDer f 2-feeding did not change plasma IgG levels in immunized mice. Neither IgG1 nor IgG2a titer showed close relationships with BALF cell count and biochemical values (Table 1). Anti-rDer f 2 IgE was also detected in sensitized mice by ELISA. However, IgE levels were not high enough to analyze in sensitized animals (Data not shown).

Effect of rDer f 2 Feeding on Allergic Inflammation in Mite-Sensitized Mice in Experiment 2

Mice immunized with rDer f 2 and mite extract in experiment 2 were challenged with mite extract. Inhalation of mite extract provoked late-phase airway inflammation characterized by neutrophil influx in mite-sensitized A/J mice. The severity of the inflammation as judged from the leukocyte count of the BALF was lower than that of the rDer f 2-induced reaction in experiment 1. The BALF neutrophil count in the PBS-fed group were $3.2 \pm 0.5 \times 10^4$ cells/ml, while that in naive mice was $0.2 \pm 0.0 \times 10^4$ cells/ml. The 0.1 and 1 mg/day rDer f 2 groups showed lower neutrophil number than control group (Fig. 4). The difference was not significant, however.

DISCUSSION

Studies in humans and experimental models have shown that oral hyposensitization therapy can suppress cellular immunity including late-phase asthmatic reaction (5, 21–26). In the present study, we examined the oral-hyposensitizing activity of rDer f 2 to mite-allergic inflammation in a murine model. Allergic neutrophil influx in rDer f 2-sensitized mice was suppressed by daily rDer f 2 feeding, suggesting the beneficial effect of oral-hyposensitization therapy with rDer f 2. rDer f 2-feeding also suppressed mite extract-induced reactions in mice immunized with mite extract

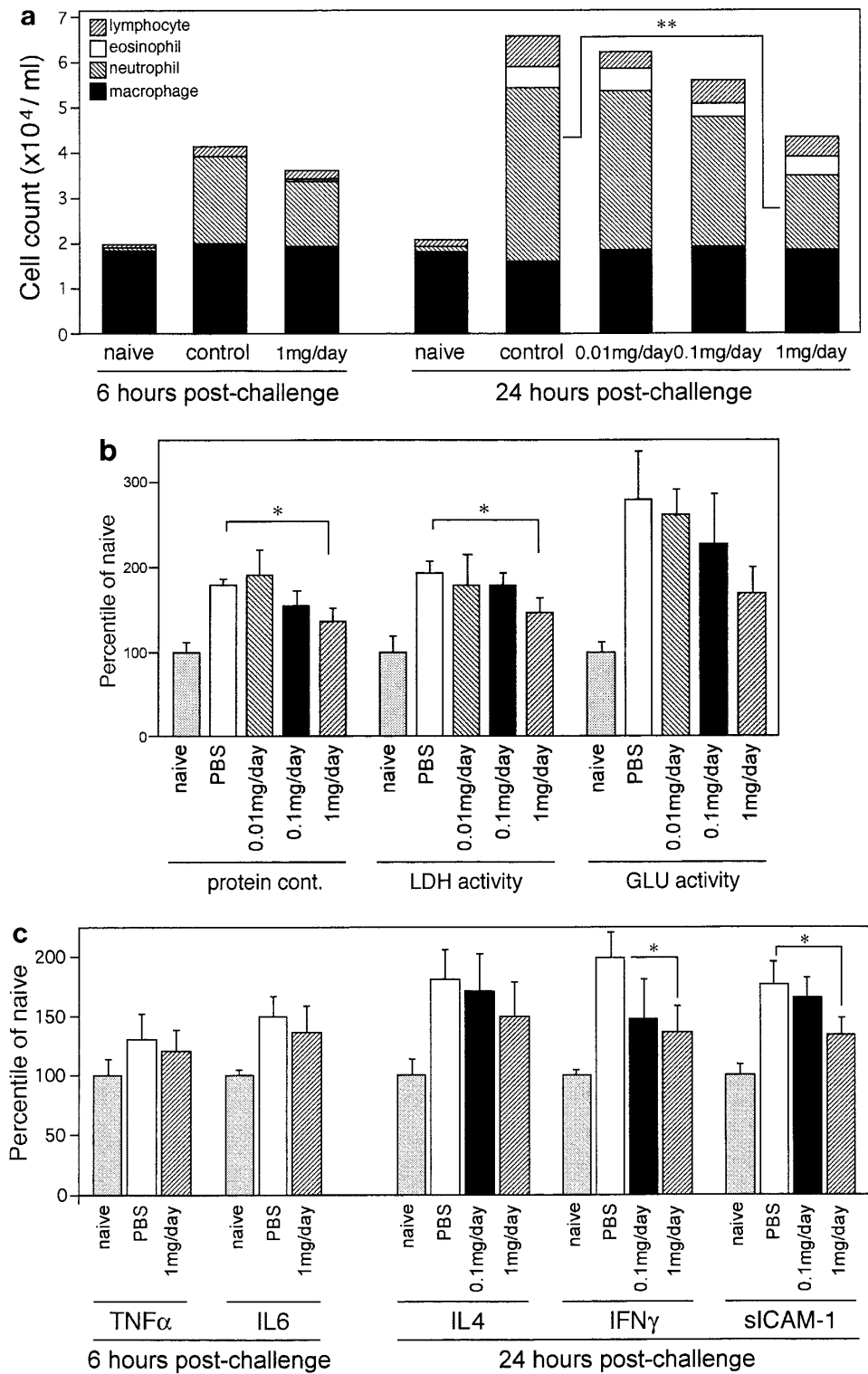


FIG. 3. The effect of oral rDer f 2 administration on (a) leukocyte counts, (b) biochemical activities, and (c) cytokines in the BALF in experiment 1. BALF was collected 6 hr after the rDer f 2 challenge from PBS-fed ($n = 7$), 1 mg/day ($n = 7$), and naive mice ($n = 6$), or 24 hr postchallenge from PBS-fed ($n = 10$), 1 mg/day ($n = 7$), 0.1 mg/day ($n = 7$), 0.01 mg/day rDer f 2-fed ($n = 10$), and naive mice ($n = 8$). ** $P < 0.01$, * $P < 0.05$.

TABLE 1

Statistical Relationships between BALF Cell Counts, Biochemical Values, and Plasma IgG Levels in Sensitized Mice

	Np	Ep	Protein	LDH	GLU	IgG1	IgG2a	IL4	IFN γ	sICAM-1
Neutrophil		0.43	0.75	0.82	0.55	0.22	-0.04	0.37	0.51	0.57
Eosinophil	ns		0.48	0.43	0.24	-0.09	0.11	-0.10	0.19	0.29
Protein	$P < 0.01$	$P < 0.05$		0.94	0.63	0.21	0.04	0.24	0.48	0.57
LDH	$P < 0.01$	ns	$P < 0.01$		0.71	0.32	-0.04	0.31	0.47	0.62
GLU	$P < 0.05$	ns	$P < 0.01$	$P < 0.01$		0.43	-0.03	0.05	0.18	0.67
IgG1	ns	ns	ns	ns	ns		-0.34	-0.01	-0.28	0.21
IgG2a	ns	ns	ns	ns	ns	ns		-0.26	0.10	-0.30
IL4	ns	ns	ns	ns	ns	ns	ns		0.59	0.18
IFN γ	$P < 0.05$	ns	$P < 0.05$	$P < 0.05$	ns	ns	ns	$P < 0.01$		0.38
sICAM-1	$P < 0.01$	ns	$P < 0.01$	$P < 0.01$	$P < 0.01$	ns	ns	ns	ns	

Note. LDH, lactate dehydrogenase; GLU, β -glucuronidase; IgG1, IgG2a, plasma levels after oral administration. Each correlation coefficient was calculated from the measurements of 20 sensitized mice in PBS and 1 mg/day rDer f 2 groups in experiment 1.

and rDer f 2. Since Der f 2 constituted only 0.3% of the provoking antigen (mite extract), a more intense sensitization with rDer f 2 might provide better results. Selection of mite-allergic patients whose major sensitizing-allergen is Der f 2 will be needed to achieve adequate levels of hyposensitization with rDer f 2.

The airway inflammation observed in the A/J mice in the present study is characterized by a severe infiltration of neutrophils into the airway. The concentration of protein, LDH, and GLU in the BALF, which could be derived from damaged cells or leaked plasma, showed a close relationship with neutrophil number. Although the role of eosinophils seems to be highlighted more than that of neutrophils in recent studies on the development of late-phase inflammation (7, 27), human and animal studies have shown that neutrophils contribute to severe airway injury and hyperresponsiveness by infiltrating inflammatory sites and releasing a variety of substances including oxygen metabolites, proteases, cationic materials, and potent lipid

mediators (14, 15). Thus inhibition of the allergic inflammation caused by neutrophils is thought to play a significant role in the efficacy of oral immunotherapy by rDer f 2.

Several studies have demonstrated a role for anergy, clonal deletion, and active suppression in oral tolerance and oral hyposensitization (22, 30, 31). We performed a kinetic analysis of cytokines and sICAM-1 in BALF to elucidate the mechanism of the immunotherapy. Neutrophil influx is very efficient under conditions of the activation of cell adhesion molecules, especially ICAM-1 in the target tissues (32). sICAM-1 levels in the BALF, which could reflect the status of the expression and activation of ICAM-1 in airway tissues (20), increased synchronously with neutrophil counts after rDer f 2 inhalation in sensitized mice over 48 hr. While the influences of IL4 and IL6 on the expression of ICAM-1 are not clear, TNF α and IFN γ have been shown to be strong inducers of ICAM-1 in various tissues in both humans and mice (33–35). Since the peak

TABLE 2

Plasma Anti-rDer f 2 IgG1 and IgG2a Levels before and after Oral Administration

	Before	After
IgG1		
PBS	1.00 $\pm$ 0.09	0.93 $\pm$ 0.12
0.01 mg/day	0.91 $\pm$ 0.07	1.07 $\pm$ 0.23
0.1 mg/day	1.17 $\pm$ 0.21	1.14 $\pm$ 0.24
1 mg/day	1.14 $\pm$ 0.14	0.93 $\pm$ 0.12
IgG2a		
PBS	1.00 $\pm$ 0.17	1.69 $\pm$ 0.38
0.01 mg/day	1.11 $\pm$ 0.32	1.55 $\pm$ 0.53
0.1 mg/day	1.21 $\pm$ 0.17	1.70 $\pm$ 0.71
1 mg/day	1.03 $\pm$ 0.27	1.30 $\pm$ 0.31

Note. Both the IgG1 and IgG2a titer of the PBS-fed group before oral treatment were about 1/4000.

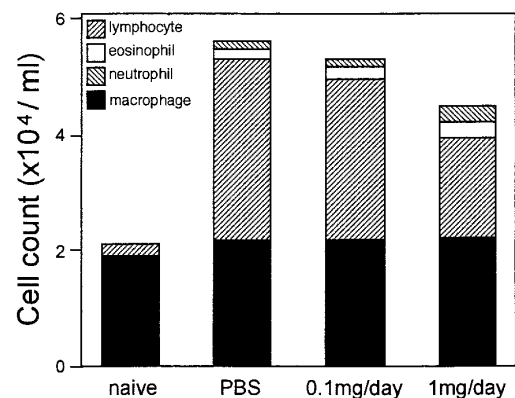


FIG. 4. The effect of oral rDer f 2 administration on leukocyte counts in the BALF collected 24 hr after inhalation of crude mite extract in experiment 2. Seven mice were examined for each group.

TNF α and IFN γ levels in BALFs (at 3 and 24 hr post-challenge, respectively) had a time lag between them, these two cytokines might share in the 48-hr-activation of ICAM-1 in this allergic model. Several studies have reported a beneficial role for IFN γ but unfavorable roles for Th2-type cytokines including IL4 and IL6 in the eosinophil-dominant late-phase reaction and immediate reaction (36, 37). On the other hand, IFN γ can play a crucial role in the pathogenesis of allergic reactions caused by cellular immunity (38, 39). It is not easy to determine which type of cytokine, Th1 or Th2, participated in the development or suppression of the complicated allergic inflammations in the present study. From the BALF, we found that all the levels of cytokines and sICAM-1 measured at 6 or 24 hr post-challenge decreased in the rDer f 2-fed animals compared to PBS-fed mice. Lower expression of cytokines could result in lower expression of adhesion molecules and less severity in leukocyte influx in the airway. It was suggested that anergy or clonal deletion played an important role in the suppression of airway inflammation by rDer f 2 feeding in rDer f 2-sensitized mice. Since both Th1 and Th2 type cytokines can induce an allergic reaction, suppression of both types at the same time is better than active suppression, in which certain kinds of cytokines are activated as "suppressors." rDer f 2 feeding did not affected the plasma anti-rDer f 2 IgG1 or IgG2a levels, or their ratio, reflecting a systemic balance between Th1 and Th2. The suppressive effects against allergic reactions by oral antigen administration might be specific for restricted tissues.

Mite extract contains many antigenic substances and is difficult to biochemically standardize (40, 41). Therefore, ideally, purified mite allergens should be substituted for mite extract in the immunotherapy of house dust mite allergy (42). The recombinant protein of Der f 2, a group 2 major mite allergen, which possesses allergic activity comparable to that of the native allergen, can be produced in large quantities (43). rDer f 2 could prove a useful clinical tool.

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