

Specific IgE and IgG1 Responses to Subtilisin Carlsberg (Alcalase) in Mice: Development of an Intratracheal Exposure Model

THOMAS T. KAWABATA¹, LAURA S. BABCOCK, AND PATRICIA A. HORN

Human Safety Department, Corporate Professional and Regulatory Services Division,
Box 538707, The Procter & Gamble Company, Cincinnati, Ohio 45253

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The primary objective of this study was to examine the feasibility of using a mouse model to evaluate the immunogenicity of proteins as a potential method to determine occupational exposure guidelines. Mice were intratracheally administered a benchmark protein allergen, subtilisin Carlsberg (Alcalase) in detergent matrix once a week for 4 to 6 weeks and specific IgE and IgG1 levels were determined. In all experiments, specific IgE levels were determined by using a rat basophilic leukemia cell (RBL) release assay, while specific IgG1 was measured by an ELISA. A good correlation was observed between IgE titers determined by the RBL assay and rat passive cutaneous anaphylaxis assay. Intratracheal administration of protease with detergent matrix was found to result in significant IgE and IgG1 responses that were dose related. Detergent matrix was found to enhance the Alcalase-specific IgE and IgG1 response when administered by the intratracheal route. The IgG1 response was much more robust, easier to measure, and found to follow the IgE response. These results suggest that a mouse intratracheal model is a feasible approach to examining the immunogenic potency of enzymes using specific IgE or IgG1 as the end points. Additional development and validation of the mouse model with other types of proteins will be pursued. © 1996 Society of Toxicology

Enzymes have been shown to produce type I respiratory allergy in workers of laundry detergent manufacturing plants (Flindt, 1969; Pepyes *et al.*, 1969; Newhouse *et al.*, 1969; Greenberg *et al.*, 1970; McMurray, 1970; Franz *et al.*, 1971; Weill *et al.*, 1971). However, stringent control of enzyme dust levels in the plants has minimized the incidence rate of sensitization (Juniper *et al.*, 1977). This safe level has been empirically established for the protease, subtilisin Carlsberg (Alcalase). As new enzymes are introduced into detergent plants, we need to evaluate the immunogenicity and compare it to the benchmark allergen, Alcalase, to determine the correct occupational exposure guidelines (OEGs) for each en-

zyme. In addition, more mechanistic information on the immunogenicity of enzymes was needed. To address these needs, the guinea pig intratracheal (GPIT) test was developed (Ritz *et al.*, 1993). In this model, guinea pigs are intratracheally (i.t.) dosed with the test enzyme and the enzyme-specific IgG1 response is used as an index of sensitization (IgG1 isotype is the major anaphylactic antibody in the guinea pig (Ovary *et al.*, 1963)). Although the GPIT test is a very useful method, it is expensive and time consuming. The unavailability of reagents for the guinea pig has also hindered studies to determine the mechanisms of enzyme-induced IgG1 production.

The goal of these studies was to determine the feasibility of using a mouse model to evaluate the immunogenicity of enzymes with the ultimate goal of developing an alternative to the GPIT test. The mouse has been used extensively to examine the IgE response. However, most of these studies involved administration of allergens via nonrelevant routes of exposure, intraperitoneal or subcutaneous. Inhalation of aerosolized antigen has been shown to be an effective approach (Renz *et al.*, 1992); however, the difficulty and costs of generating and quantitating aerosols make this an impractical approach to evaluating quickly the immunogenicity of various types of enzymes at multiple exposure levels. Intratracheal administration may be a more feasible method. Ritz *et al.* (1993) have demonstrated in the guinea pig that exposure to similar doses of Alcalase by the i.t. and inhalation routes of exposure yielded similar levels of specific IgG1 antibody. A few studies using mice have demonstrated that i.t. (Holt *et al.*, 1981; Tamura *et al.*, 1986; Fujimaki *et al.*, 1986a,b) administration of antigen was able to elicit a measurable IgE and IgG response. However, it was not clear from these reports which of these antibody isotypes would be the most useful in a mouse model as a replacement to the GPIT.

The first objective of this study was to evaluate the IgE and IgG1 response in serum of mice administered a protease by the i.t. route. Since detergent plant workers are exposed to airborne enzymes in a detergent matrix, the second objective was to examine the role of detergent matrix in the spe-

¹ To whom correspondence should be addressed.

cific IgE and IgG1 response to enzyme with i.t. exposure. The results of these studies demonstrate that the i.t. route of administration is an effective method in producing a dose-dependent IgE and IgG1 response in Balb/c mice that is enhanced by coexposure to detergent matrix.

MATERIALS AND METHODS

Animals. Female Balb/c mice (18–20 g) were obtained from Charles River (Portage, MI). Mice were housed in cages with wood chip bedding. The animals were kept in rooms controlled for humidity (30–70%) and temperature (67–77°F) and with 12-hr light and dark cycles. Animals were allowed feed (Purina Chow) and water *ad libitum*. All animal treatments were done in accordance with procedures approved by the Institutional Animal Care and Use Committee.

Chemicals. Alcalase was obtained from Novo Industri A/S (Bagsvaerd, Denmark) and contained 33.6% protein (Kjeldahl nitrogen analysis). The detergent matrix used was formulated by the Procter & Gamble Co. and consisted primarily of anionic and nonionic surfactants, silicate builders, and perborate bleach.

Dosing methods. Mice were i.t. dosed by using a modification of the methods reported by Starcher and Williams (1989) and Sikorski *et al.* (1989). Mice were anesthetized with an ip injection of Ketaset (88.8 mg/kg) and Rompun (6.67 mg/kg). Mice were placed on a Plexiglas restrainer (30° angle). Using a fiber optic light (Cole Palmer, Chicago, IL) taped to a tongue depressor to illuminate the epiglottis, a 23-gauge (blunt) angled needle attached to a 500- μ l Hamilton glass syringe was inserted past the epiglottis and into the trachea. To confirm that the needle was in the trachea, the plunger of the syringe was gently pulled back. Free movement of the plunger indicated that the needle was in the trachea. Once in the trachea, the 500- μ l syringe was replaced with a 250- μ l syringe filled with the dosing solution (100 μ l). The solution was injected into the trachea. The syringe was removed and 100 μ l of air was subsequently injected to push the remaining dosing solution into the lung.

Mice were i.t. dosed once a week for 4 to 6 consecutive weeks. Five days after the last dose, mice were anesthetized by inhalation of Metofane and exsanguinated. In preliminary studies, it was determined that the specific IgE and IgG1 responses in serum peaked 5 days following the final exposure (data not presented).

Rat basophilic leukemia (RBL) release assay. Alcalase-specific IgE in mouse serum was measured by the method described by Kawabata and Babcock (1993). Briefly, cells (RBL-2H3, 1×10^5 /well) were incubated overnight with [³H]serotonin (NEN, Boston, MA, 0.1 μ Ci/well) in 96-well plates. Media were removed and replaced with 100 μ l of twofold dilutions of the test sera (in fresh media) and incubated at 36.4°C for 3.5 hr in 5% CO₂. After incubation, media containing unbound antibody was removed, 100 μ l of Alcalase (2 μ g/well) was added to the wells, and the plates were incubated for 45 min as described above. Media were collected (released) and 20 μ l was added to 200 μ l of scintillation cocktail (Packard, Microscint 40). The samples were counted for 5 min. To the remaining cell monolayer was added 100 μ l of 1% Triton-X 100. Cells were lysed and 20 μ l was removed for counting (unreleased). Appropriate controls [e.g., normal sera (obtained in-house), mouse α DNP and DNP–human serum albumin (Sigma Chemicals, St. Louis, MO)] were included in each experiment.

The percentage [³H]serotonin released was calculated by dividing the dpm released by the total dpm of serotonin incorporated (released + unreleased) and multiplied by 100. Specific IgE titer is defined as the highest dilution of test sample with a percentage release value greater than or equal to the 2 $\times$ the percentage released of sample exposed to normal serum and antigen. The range of the average percentage release for sample dilutions ranged from 15 to 30%, while the various negative controls were <10%. The mouse anti-DNP + DNP antigen (positive control) elicited an average

of 40% serotonin release. Throughout this report, specific titer was presented as the log₂ of the reciprocal dilution.

Rat PCA assay. The rat PCA was used to validate the RBL assay in measuring Alcalase-specific IgE as described by Kawabata and Babcock in 1993. The PCA assay was conducted according to the method of Ovary *et al.* (1975). Rats were intradermally administered 0.1 ml of twofold serial dilutions of mouse test sera. Twenty-four hours later the animals were given an iv injection of Alcalase (2 mg/rat) in 1% Evan's Blue dye (Sigma). The reciprocal of the highest dilution yielding a blue mark was considered to be the titer.

Specific IgG1 ELISA. Alcalase-specific IgG1 in mouse serum was measured by using an ELISA. One hundred microliters of Alcalase at 10 μ g/ml in Dulbecco's phosphate-buffered saline (DPBS; Sigma) was added to wells of Immunlon 2 microtiter plates (Dynatech, Chantilly, VA) and incubated overnight at 4°C. The Alcalase solution was removed and replaced with 200 μ l Wash Buffer (DPBS with 0.05% Tween 20) and incubated at room temperature for 1 hr. The Wash Buffer also served as the "blocking buffer" to prevent nonspecific binding of immunoglobulins. Test serum (serially diluted in Wash Buffer with 1% bovine serum albumin) was added to microtiter plates and incubated for 1 hr at 37°C. Plates were washed 3 $\times$ with wash buffer. Goat anti-mouse IgG1-AP conjugated antibody (Southern Biotechnology, Birmingham, AL) was added (100 μ l) to each well and incubated for 1 hr at 37°C. *p*-Nitrophenyl phosphate substrate dissolved in diethanolamine buffer was used (Kirkegaard and Perry, Gaithersburg, MD) and read with a Thermomax microplate reader (Molecular Devices, Menlo Park, CA) at 405/650 nm.

Alcalase-specific IgG1 titers were determined by using the method described by Peterman (1991) and Butler and Hamilton (1991). In this method, the data was plotted in a log dilution vs. log optical density plot. The O.D. determined from normal mouse serum was subtracted from O.D. values of test serum. A linear regression analysis on the linear region (antibody limiting) of the curve was performed using Softmax software (Molecular Devices). Antibody titer was expressed as the log₂ value of the reciprocal dilution produced at an OD of 0.5 (determined by interpolation of the line).

Mouse bronchial alveolar lavage (BAL). Mice were anesthetized as described above. The anesthetized mice received various concentrations of Alcalase intratracheally. Eighteen to 24 hr after instillation with Alcalase the mice were euthanized with a lethal ip injection of sodium pentobarbital. The trachea was surgically exposed and tubing (connected to a two-way stopcock with attached 3-ml syringes) was inserted. With the tubing secured within the trachea, the lungs of the mice were gently massaged with 0.5 ml of saline, with a total of 2 ml used. Recovered volumes containing cells ranged from 1.2 to 1.8 ml. Cell suspensions were held on ice, washed 2 $\times$ in cold PBS, and resuspended in 1ml PBS with 2% FCS for counting. Suspensions were counted using a Coulter ZM Counter (Hialeah, FL) and diluted to 1×10^5 cells/ml. A 0.5-ml sample of this dilution was centrifuged onto glass slides using the Shandon Cytospin (Pittsburg, PA). Slides were airdried overnight and stained with Wrights Stain (Sigma). Differentials were performed on the stained slides.

RESULTS

Measurement of Alcalase-Specific IgE

Levels of Alcalase-specific IgE were measured in serum by the RBL release assay. The original method was validated using mouse anti-ovalbumin serum (Kawabata and Babcock, 1993). To determine if the RBL method is also able to measure Alcalase-specific IgE, titers determined by the RBL assay were compared to those determined by the rat PCA assay. Samples with a wide range of titers were examined. A good correlation was observed between the two methods

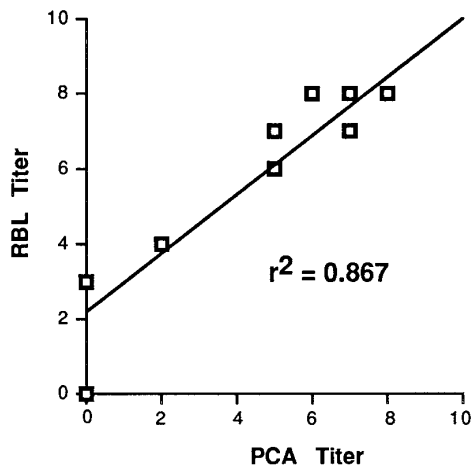


FIG. 1. Comparison of Alcalase-specific IgE titers determined by the RBL and PCA assay. Alcalase-specific titers from 11 sera were measured in the RBL Release Assay and the rat PCA. The correlation coefficient (r^2) was calculated to be 0.867.

($r^2 = 0.867$) (Fig. 1). Thus, specific IgE titers of serum samples for the study were determined by the RBL release assay.

Comparison of Dosing Regimen and Routes of Administration

The appropriate i.t. dose of Alcalase for sensitization was determined by examining the inflammatory response in the lung. Cell types in BAL samples of mice i.t. dosed with Alcalase were examined. A dose of 1.0 $\mu\text{g}/\text{mouse}$ produced a significant increase in total cell numbers (Table 1). This

increase was attributed to an increase in neutrophils. Based on these results, doses less than 1.0 $\mu\text{g}/\text{mouse}$ was used for sensitization. In comparison to Alcalase, the detergent matrix at doses up to 500 $\mu\text{g}/20\text{ g mouse}$ did not dramatically alter total cell number or individual cell types (Table 1). For the remaining experiments, a dose of 100 μg detergent matrix/20g mouse was used since most detergent formulations contain approximately 0.1–1.0% enzyme.

Two sensitization regimens were examined for the effectiveness of inducing levels of specific IgE in serum. Mice were primed with an ip dose of Alcalase and alum followed by three or five weekly i.t. doses of Alcalase plus detergent matrix (Fig. 2A). A significant IgE response was observed using this sensitization regimen. The second dosing regimen involved weekly i.t. doses (2–8 weeks) of Alcalase plus detergent matrix (Fig. 2B). The titer increased with increasing number of doses. Maximal IgE responses were observed after 6 weeks of dosing that was equivalent to the levels obtained with ip priming and i.t. boosting.

Effect of Anesthetic Treatment on the IgE Response

To determine if the anesthetic used during intratracheal dosing was altering the IgE response to Alcalase, a study was conducted to determine the IgE response in unanesthetized and anesthetized mice. Since mice must be anesthetized for i.t. dosing, this study used the ip route of sensitization. Mice received ip injections of 30 μg Alcalase and 4 mg Alum in PBS with or without a prior ip administration of anesthetic (Ketaset/Rompun cocktail). Animals were dosed once per week for 4 consecutive weeks. Sera were collected 5 days after the final dose. Alcalase-specific IgE titers of

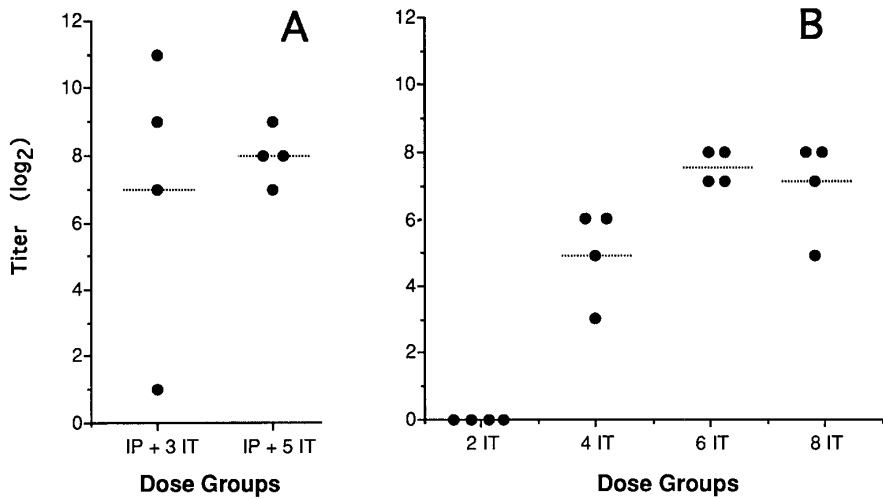


FIG. 2. Specific IgE responses produced with ip + i.t. and i.t.-only dosing. Groups of mice received one ip priming injection (10 μg Alcalase + 4 mg alum), followed by 3 or 5 i.t. (0.3 μg Alcalase and 100 μg detergent matrix) doses (A). Another group of mice received 2, 4, 6, or 8 i.t. doses without ip priming (B). All doses were administered weekly, followed by collection of sera 5 days after the last dose. Each value represents the titer of single animal. Each horizontal bar represents the mean IgE titer.

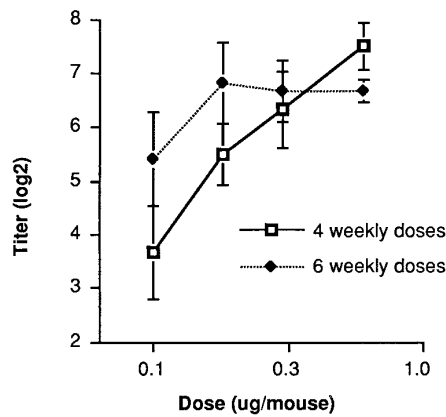


FIG. 3. Relationship between Alcalase dose to IgE titer in i.t.-dosed mice. Mice (5/group) received 0.1, 0.18, 0.3, or 0.6 μ g Alcalase and 100 μ g detergent matrix in PBS/20 g mouse i.t. for 4 or 6 weeks. Sera was collected 5 days after the final dose. Each value represent the mean $\pm$ SE.

unanesthetized mice (8 ± 0.22) did not significantly differ from those of anesthetized mice (7.4 ± 0.66).

Dose-related IgE and IgG1 Responses in i.t. Dosed Mice

Groups of mice were administered different doses of Alcalase in the presence of detergent matrix for 4 and 6 weeks (Fig. 3). A dose-related increase in specific IgE titers was

TABLE 1

Determining the Appropriate Dose of Alcalase and Detergent Matrix by Examining the Inflammatory Response of i.t. Dosed Mice: Bronchial Alveolar Lavage Studies

Dose ^a	Cell No. ($\times 10^4$)			
	Total cell count	Macrophages	Lymphocytes	Neutrophils
Alcalase				
No treatment	58.9	57	1	1
0	69.7	29	0	41
0.1	79.1	33	2	43
0.3	61.7	44	1	17
1.0	293.1	53	3	185
Detergent matrix				
0	97.4	73	17	7
50	56	51	1	4
250	67	49	0	18
500	113.2	80	3	36

Note. Mice received a single i.t. dose of Alcalase or detergent matrix in 0.9% sodium chloride. The vehicle control is indicated by the "0 dose" group. The "no treatment group" received a sham treatment—placement of needle in trachea without an injection. Cells from bronchial alveolar lavaged mice were obtained 16 to 18 hr after the i.t. dose. The cell types were enumerated from stained cytospin slides. Each value represents the mean of two mice.

^a Alcalase dose in μ g/20 g mouse.

observed with 4 weeks of dosing. Six weekly doses produced a slightly greater IgE response than four weekly exposures at the lower doses (0.1 and 0.18 μ g/mouse). The IgE response plateaued at the higher doses and did not differ from IgE titers obtained with four weekly doses. Specific IgE and IgG1 titers were also compared in another study in which mice were administered four weekly i.t. doses of Alcalase. Specific IgG1 titers were higher than IgE titers but followed a similar dose-response relationship (Fig. 4). This was also observed in the study described in the next section (Fig. 5).

Effects of Detergent Matrix on the IgE Response to Alcalase

Alcalase administration alone by the i.t. route produces a weak response at a dose of 0.3 μ g/mouse (Fig. 5). Coadministration of Alcalase with detergent matrix dramatically enhanced specific IgE and IgG1 antibody titers. At the lower doses of Alcalase, a dose of 100 μ g detergent matrix/mouse produced a greater enhancement than the 30 μ g/mouse dose.

DISCUSSION

The primary objective of this study was to determine the feasibility of using a mouse model as a replacement to the GPIT test to assess the immunogenicity of inhaled enzymes. The effectiveness of administering enzymes via the trachea in the Balb/c mouse to produce specific IgE and IgG1 responses was investigated. The need to quickly analyze numerous serum samples for specific IgE titers required an alternative to the time- and animal-consuming rat PCA. The *in vitro* RBL release assay was used and found to be a valid alternative to the rat PCA assay in determining Alcalase-specific IgE.

The results of this study demonstrate that the i.t. route of

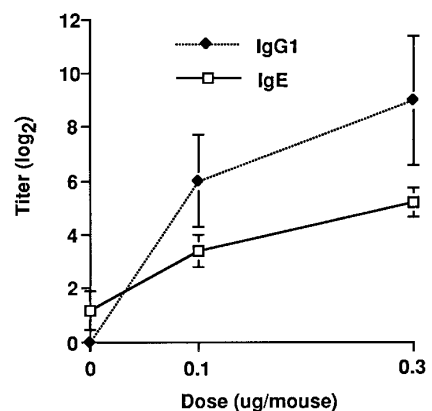


FIG. 4. Alcalase dose-response can be measured by two end points. Antigen-specific IgG1 is measured by ELISA and antigen-specific IgE is measured by the RBL release assay. Both end points have comparable curves shapes and demonstrate the effect of various concentrations of Alcalase on the immune response.

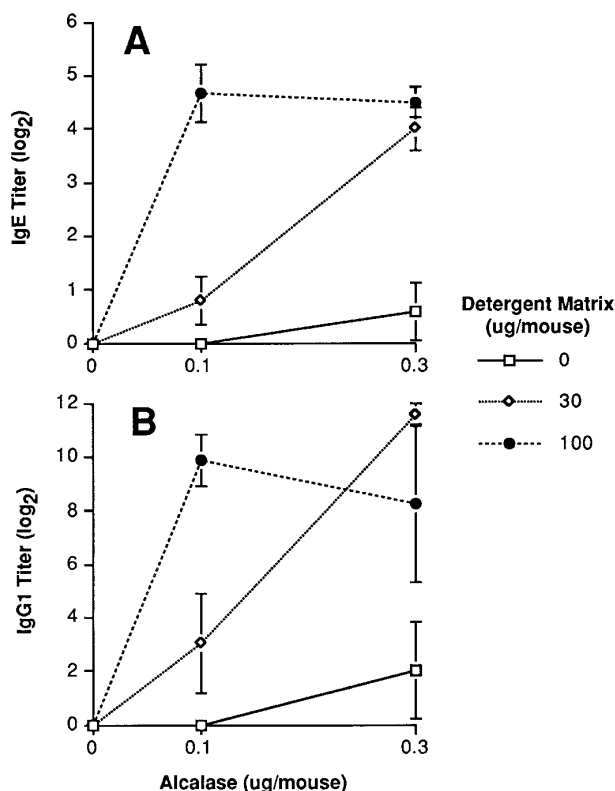


FIG. 5. Effects of detergent matrix on the IgE response to Alcalase. Mice received either 0.1 or 0.3 μ g Alcalase/20 gm mouse for 4 weeks by the i.t. route. All doses of Alcalase were with 0, 30, or 100 μ g of detergent matrix. Each value represent the mean $\pm$ SE.

exposure is able to elicit a strong IgE response to Alcalase, equivalent to that produced with ip priming and i.t. boosting. Specific IgE levels in serum were directly proportional to the dose of Alcalase. With repeated i.t. exposures, the IgE and IgG1 responses began to plateau. This was also observed for the specific serum IgG1 response in guinea pigs exposed to Alcalase via the i.t. and inhalation routes (Ritz *et al.*, 1993).

The IgE and IgG1 response to Alcalase was found to be dramatically enhanced with coexposure to detergent matrix. This is consistent with the enhanced antibody response observed in guinea pigs exposed to inactivated subtilopeptidase A (protease from *Bacillus subtilis*) and coaerosol exposure to anionic detergent (Markham and Wilkie, 1976). A study in Cynomolgus monkeys exposed by inhalation to Alcalase demonstrated enhanced levels of precipitating antibody with coexposure to detergent matrix (Cashner *et al.*, 1980). The mechanism of the enhancing effects of detergent matrix on the antibody response is not clear at this time. Studies will be conducted to determine the mechanism and component(s) of the matrix primarily responsible for the enhancing effects.

Others have demonstrated that a significant IgE and IgG1 response can be produced with i.t. administration of antigen.

However, since different antigens and adjuvants were used, it is difficult to compare responses observed in this study with other reports. Intratracheal administration of tetanus toxoid with heat-killed *Bordetella pertussis* cells as an adjuvant followed by an i.t. boost of tetanus toxoid alone was also found to produce a significant serum IgE response (Gerbrandy and Bienenstock, 1976). The IgE response of tetanus toxoid without adjuvant was not examined in this study (Gerbrandy and Bienenstock, 1976). Fujimaki *et al.* (1986a,b) reported that intratracheal administration of ovalbumin did not produce a measurable primary response in mice. However, when ovalbumin was coadministered with fly ash or aluminum silicate, a significant primary IgE response was observed in Balb/c mice. In contrast, crude extract from Japanese cedar pollen without the use of adjuvant was found to produce a strong specific IgE response after three i.t. exposures in Balb/c, DBA/2, and BDF1 mice (Tamura *et al.*, 1986). A weak response was also observed after primary sensitization.

The results of this study demonstrate that the i.t. route of exposure is a feasible method for examining the immunogenicity of enzymes in mice. The specific IgG1 response to Alcalase followed the IgE dose-response relationship. However, the specific IgG1 responses were much more robust and easier to measure than IgE responses (ELISA vs RBL). Thus, the IgG1 response may be used as the end point in examining the immunogenicity of enzymes. It is not known if the antibody responses generated with the i.t. route would be similar to that produced with inhalation exposure at comparable doses. However, it is known that similar antibody responses to Alcalase via each exposure route were observed in the guinea pig (Ritz *et al.*, 1993). Future studies will compare the IgG1 and IgE response of Alcalase to other enzymes administered by the i.t. route.

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