

Specific Antibody Responses to Subtilisin Carlsberg (Alcalase) in Mice: Development of an Intranasal Exposure Model¹

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An intranasal (i.n.) dosing model was developed in mice as a potential alternative to more difficult, time-consuming, and costly guinea pig intratracheal (GPIT) or mouse intratracheal models for assessment of the respiratory immunogenicity of detergent enzymes. Using a benchmark enzyme, Alcalase (protease subtilisin Carlsberg), studies were conducted to standardize the model in terms of mouse strain, dosing and serum harvest regimen, and the primary immunoglobulin endpoint to use. The primary assay endpoint selected was the enzyme-specific IgG1 titer determined by an Alcalase-specific ELISA. This is not the primary allergenic antibody in mice (IgE is); however, IgG1 is coregulated with IgE via the IL-4/TH2 pathway and may have a role in mediating allergic-type responses. BDF1 mice (C57Bl/6 × DBA/2) were selected as representative of high responder strains, with high response associated with the H-2^b (C57Bl/6) parent. The dosing regimen used for most studies incorporated three i.n. exposures (Days 1, 3, and 10) and bleeding of the animals on Day 15. The animals were anesthetized and then immunized by allowing them to inhale 5- μ l aliquots of dosing solution into each nostril at each immunization. Positioning of the animals with their heads down (vs up) may have allowed more of the dosing solution to remain in the nasal region for a slightly longer period of time, but did not change the eventual GI tract migration and excretion of each dose. The presence of a detergent matrix in the enzyme dosing solution enhanced the IgG1 response. Immunizing with enzyme plus detergent gave highly consistent dose–response curves for Alcalase when evaluated over many studies. An enzyme-specific allergic antibody (IgE) response was weak and inconsistent under the dosing regimen used to generate the IgG1 response, but was stronger with longer-term dosing, consistent with the delay in IgE vs IgG1 responses seen in some other studies. Using IgG1 as a surrogate for allergic sensitization, we have preliminary data showing similar differential potencies between Alcalase and other test enzymes as detected in previous GPIT tests. On the basis of these data, we

believe the i.n. immunization/IgG1 response model is a robust technique that may be useful in determining the relative immunogenicities of detergent enzymes and other proteins. © 1996 Society of Toxicology.

The application of threshold limit values (TLV)² and operational exposure guidelines for detergent enzymes has minimized the incidence rate of sensitization for Type I respiratory hypersensitivity. The TLV of the most widely used enzyme, Alcalase (protease subtilisin Carlsberg), was set empirically in the manufacturing plants. To determine the operational guidelines for new enzymes that may be more or less allergenic than Alcalase, a guinea pig intratracheal test (GPIT) was developed (Ritz *et al.*, 1993). In this assay, guinea pigs are administered either test enzyme or Alcalase once per week for 10 weeks and the enzyme-specific IgG1 responses are measured at different time points. IgG1 is the major allergic antibody in the guinea pig (Sarlo and Karol, 1994). Thus, the relative allergenicity of a test enzyme is determined by comparing the response (serum IgG1 antibody titer) of the test enzyme with that produced by the benchmark, Alcalase (Sarlo *et al.*, 1991). The GPIT test is a very useful method for the evaluation of enzyme allergenic potential; however, it is also very time consuming and expensive. Therefore, our lab has focused on developing quicker and less expensive alternative methods.

Important criteria for an alternative method are that a specific antibody response must develop within 3–4 weeks and that it be robust and easily measured. In addition, the method should be relatively inexpensive, should not require highly specialized equipment, and therefore be readily transferable to other labs. Although significant antibody responses to proteins (including enzymes) can be obtained with subcutaneous or intraperitoneal routes of sensitization when used

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² Abbreviations used: BSA, bovine serum albumin; DPBS, Dulbecco's phosphate-buffered saline; dpm, disintegrations per minute; ED50, effective dose for 50% of maximum response; ELISA, enzyme-linked immunosorbent assay; GPIT, guinea pig intratracheal test; i.n., intranasal; i.t., intratracheal; OD, optical density; TLV, threshold limit values.

with or without adjuvants such as alum (Taylor *et al.*, 1980; Holt *et al.*, 1981b; Vaz *et al.*, 1971; Beck and Spiegelberg, 1989; Hilton *et al.*, 1994), we decided to evaluate routes of exposure more relevant for respiratory hypersensitivity.

We initially investigated the feasibility of a mouse model in which the test enzyme was administered by the intratracheal (i.t.) route (Kawabata *et al.*, 1996). We were able to demonstrate that, after 4 weekly doses of Alcalase (admixed with detergent matrix), significant dose-dependent specific IgE and IgG1 responses were produced. Specific IgE was measured by an *in vitro* RBL serotonin release assay (Kawabata and Babcock, 1993) and specific IgG1 was measured by an antigen-capture ELISA. Based on initial results, the i.t. method appeared to be a feasible alternative to the GPIT test. However, before conducting numerous enzyme potency comparison studies, we decided to examine the intranasal (i.n.) route of administration. Not only has the i.n. route of administration been commonly used to immunize mice to a variety of viral (Muller and Brons, 1994; Walsh, 1994), bacterial (Garcia *et al.*, 1993, 1994), fungal (Wang *et al.*, 1994), and soluble protein (Takafuji *et al.*, 1989; Henderson *et al.*, 1985) antigens, but the methodology was much easier to conduct than the i.t. dosing regimen.

Others have demonstrated that i.n. administration of proteins to mice (Takafuji *et al.*, 1989; Henderson *et al.*, 1985; Inagaki *et al.*, 1985; McCaskill *et al.*, 1982) and rats (Hamelers *et al.*, 1991) is able to elicit specific IgE and/or IgG responses. However, studies were needed to determine the feasibility of using an i.n. route of administration in mice as an alternative test for measuring systemic antibody responses to enzymes. The specific objectives of this study were to evaluate the antigen-specific IgG1 and IgE antibody responses to i.n.-administered Alcalase and to optimize the method for determining relative enzyme potencies. Antibody responses of different mouse strains were examined along with various dosing regimens. In addition, we investigated the kinetics of response, the effects of detergent matrix on the Alcalase-specific antibody response, and the kinetics of disposition of i.n.-dosed ¹²⁵I-labeled bovine sera albumin (BSA) in various tissues. We have also compared the differential potency results between Alcalase and test enzymes with earlier results generated in the GPIT test.

We were able to detect consistent enzyme-specific anti-Alcalase IgG1 responses after three intranasal exposures of mice, over 10 days, to enzyme in saline or detergent matrix. Full dose-response profiles were obtained. Though the IgG1 response might be considered primarily an indicator of enzyme *immunogenicity*, IgG1 is known to be coregulated with IgE in the mouse via the Th2/IL-4 pathway (Purkerson and Isakson, 1992) and has been associated with allergic responses in IgE-deficient mice (Oettgen *et al.*, 1994; Lei *et al.*, 1996) and in passively sensitized mice (Oshiba *et al.*,

1996). Thus, IgG1 is a potential surrogate marker for assessing enzyme *allergenicity*. The results of these studies demonstrate that the i.n. dosing method and IgG1 antibody assay is a highly practical and robust method for assessing enzyme potency and represents a useful alternative to costlier, more time-consuming guinea pig and mouse intratracheal test methods.

MATERIALS AND METHODS

Animals

Female mice (~18–20 g) of various strains were obtained from Charles River (Portage, Michigan). The mice were housed in cages with wood chip bedding. The animals were kept in rooms controlled for humidity (30–70%), temperature (67–77°F), and 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 an Institutional Animal Care and Use Committee. Four or five animals were included in each dosing group across all studies reported.

Test Chemicals

Alcalase (protease subtilisin Carlsberg) was obtained from Novo Industri A/S (Bagsvaerd, Denmark) and contained 33.6% protein (Kjeldahl nitrogen analysis). Alcalase was diluted (on a protein weight basis) in 0.9% sodium chloride alone or containing a detergent matrix. The detergent matrix used was formulated by Procter & Gamble and consisted primarily of anionic and nonionic surfactants, silicate builders, and perborate bleach.

Intranasal Dosing Method Development

Volume and position. Animals were anesthetized by an intraperitoneal (ip) injection of a mixture of Ketaset (88.8 mg/kg) and Rompun (6.67 mg/kg). The extent of anesthesia was determined based on desired time to recovery (approximately 10 min) and lack of any anesthesia-related deaths and was carefully controlled through the amount (by weight) administered. The animals were held in the palm of the hand, backs down. Animals were dosed intranasally (held upright or in a slight downward position) with various concentrations of enzyme (0.01–20 µg protein) in saline with or without detergent matrix. Dosing solutions were administered in volumes of 10 or 60 µl/mouse. The dosing solutions were divided equally between the two nostrils (i.e., 5 or 30 µl/nostril). The dosing solutions were gently placed on the outside of each nostril and inhaled by the mouse. Following administration of the enzyme solutions, the animals were placed on either their stomachs or their backs upon a mound of slightly angled bedding.

Determination of optimal intranasal dosing regimen and peak antibody response. Animals were anesthetized and dosed as described above. Animals generally received doses of Alcalase on Days 1, 3, and 10. Collection of sera was performed generally 5 days after the last instillation of antigen. Additional studies were conducted to confirm the optimal time to collect the sera from the mice. Mice were dosed i.n. with 0.18, 0.3, or 0.6 µg Alcalase protein on Days 1, 3, 10. Sera were collected from the animals on Days 3, 4, 5, 6, and 7 post final administration of antigen. All sera were analyzed for IgG1 titers. In one experiment, the submandibular lymph nodes were excised at the time of bleeding and were processed for total cell counts. An extended dosing regimen was also conducted with sera analyzed for both IgG1 and IgE titers. Six groups of mice were dosed i.n. with 0.5 µg Alcalase protein on Days 1, 3, and 10. Groups 2–6 were given two to six additional weekly doses. All mice were bled on Day 5 after the final i.n. dose of Alcalase.

Effect of detergent matrix on the immune response. The mice were dosed with different concentrations of Alcalase (0.1–20 μg) along with various concentrations of detergent matrix (0, 10, 30, and 100 μg). Animals received dosing solutions on Days 1, 3, and 10. Sera were collected 5 days after the last instillation.

Histopathology of the Nasal Mucosa

Two doses of Alcalase (0.03 and 1.0 μg) with 30 μg detergent matrix (or detergent matrix in saline only) were administered i.n. (5 μl /nostril). Test materials were administered on Days 1, 3, and 10 with euthanization on Day 15. At necropsy, nasal tissues were collected (Pathology Associates, Inc., West Chester, OH) in 10% buffered Formalin and select tissues were decalcified and prepared for microslide preparation. Each nose was trimmed so that microslides displayed three transverse sections, including all major nasal structures and epithelial surfaces. Microslide evaluations were conducted without knowledge of the specific treatment modalities (i.e., in a blind manner).

Distribution of Intranasal Dosing Solutions Using ^{125}I -BSA

Animals were dosed with 1 μg BSA conjugated to ^{125}I (1 μCi ; ICN Biomedicals, Inc., Irvine, CA) containing 30 μg detergent matrix. Two different volumes (10 and 60 μl) were administered. Also, animals that received 10 μl were dosed in either a head up or head down position. Animals were euthanized at 30 min. and 2, 6, 24, 48, 72, and 96 hr after instillation of ^{125}I -BSA. Following euthanization, blood, trachea, esophagus, lungs, stomach, liver, kidneys and spleen, mouth area, nasal area, head, intestines, and the remaining carcass were placed into scintillation vials and counted (Minaxi, Packard).

Rat Basophilic Leukemia (RBL) Release Assay

Alcalase-specific IgE in mouse serum was measured by a previously described method (Kawabata and Babcock, 1993; Kawabata *et al.*, 1996). Briefly, cells (RBL-2H3, 1×10^5 /well) were incubated overnight with [^3H]-serotonin (0.1 μCi /well; NEN, Boston, MA) in 96-well plates. Medium was removed and replaced with 100 μl of twofold dilutions of the test sera (in fresh medium) and incubated at 36.4°C for 3.5 hr in 5% CO_2 . After incubation, medium containing unbound antibody was removed and 100 μl of Alcalase (2 μg /well) was added to the wells and incubated for 45 min as described above. Medium was collected (released label) from each well and 20 μl was added to 200 μl of Microscint 40 scintillation cocktail (Packard, Meridian, CT). The samples were counted (TopCount; Packard) for 5 min. Then 100 μl of 1% Triton X-100 was added to the remaining cell monolayer. Cells were lysed and 20 μl of the lysate was removed for counting (unreleased label). Appropriate controls [e.g., normal sera (obtained in-house), mouse anti-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 released dpm by the total dpm of serotonin incorporated (released dpm + unreleased dpm) and multiplying by 100. Specific IgE titer is defined as the highest dilution of test sample with a percentage release value greater than or equal to two times the percentage released dpm of cells exposed to normal serum and antigen. Titers were expressed as $\log_2$ of the reciprocal dilution.

Specific IgG1 ELISA

Enzyme-specific IgG1 in mouse serum was measured by using an antigen capture ELISA as previously described (Kawabata *et al.*, 1996). One hundred microliters of enzyme at 10 $\mu\text{g}/\text{ml}$ in Dulbecco's phosphate-buffered saline (DPBS; Sigma) was added to wells of Immulon 2 microtiter plates (Dynatech, Chantilly, VA) and incubated overnight at 4°C. The enzyme

solution was removed and replaced with 200 μl 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 three times with wash buffer. Alkaline phosphatase-conjugated goat anti-mouse IgG1 antibody (IgG1-AP) (Southern Biotechnology, Birmingham, AL) was added (100 μl) to each well and incubated for 1 hr at 37°C. *p*-Nitrophenylphosphate dissolved in diethanolamine buffer (Kirkegaard and Perry, Gaithersburg, MD) was added as substrate at 100 μl per well. The plates were incubated at 37°C for 15 min and read with a Thermomax microplate reader (Molecular Devices, Menlo Park, CA) at 405/650 nm.

The methods of Butler and Hamilton (1991) were used to calculate titer from ELISA data and have been described previously (Kawabata *et al.*, 1995a, 1996). Briefly, this involved the use of Softmax software (Molecular Devices). For each dilution series of test sera, the optical density (OD) of normal sera at a 1:8 dilution was subtracted from the OD of the test sera. A plot of the titration curve for the test sera was created using a log–log curve fit. Removal of data points from outside the linear portion of the curve resulted in slopes ranging from (–0.6) to (–1.2). At least one point on the edited curve contained an absorbance value of ≥ 0.5 . The serum dilution at an absorbance of 0.5 OD was calculated by interpolation on the generated line. Titers were expressed as $\log_2$ of the reciprocal dilution.

Data Analysis

In some cases, the antibody titers were analyzed for statistical significance using the two-sided Student *t* test. A *p* value of <0.05 was considered statistically significant. In certain studies, ED50 values were determined from the IgG1 response versus enzyme dose curve. These were determined using SigmaPlot logistics curve fitting software (SigmaPlot for Windows, Version 2.0, Jandel Scientific, San Rafael, CA).

RESULTS

Dose Response to Alcalase

A short-term i.n. immunization and serum collection regimen was selected for evaluating the IgG1 response to Alca-

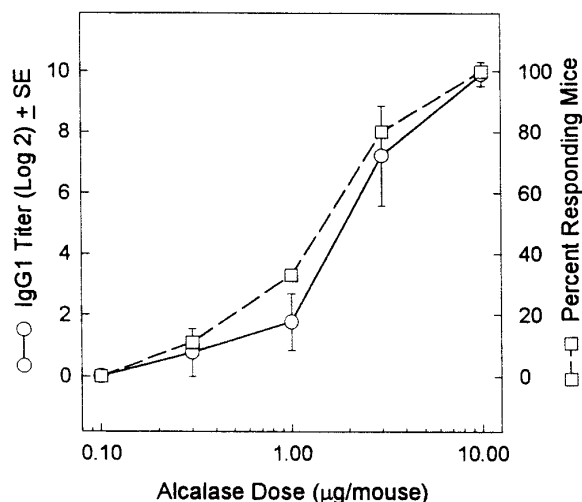


FIG. 1. BDF1 mice were administered alcalase i.n. in saline on Days 1, 3, and 10 at the indicated concentrations. Sera were obtained on Day 15 and assayed for alcalase-specific IgG1 by ELISA (Kawabata *et al.*, 1996).

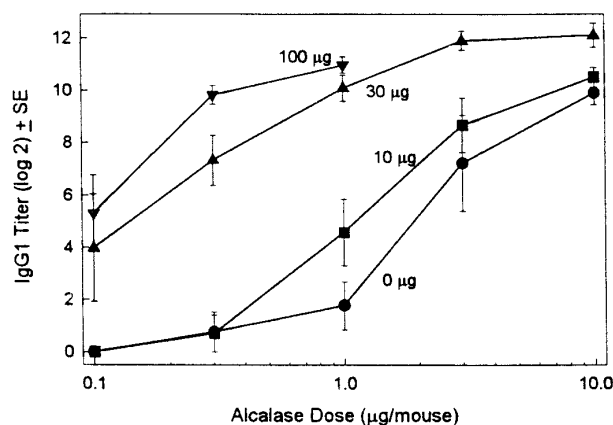


FIG. 2. BDF1 mice were administered alcalase i.n. on Days 1, 3, and 10 at the indicated concentrations. The alcalase was administered in saline or saline containing the indicated amounts of detergent (see Materials and Methods for detergent composition). Sera were obtained on Day 15 and assayed for alcalase-specific IgG1 by ELISA (Kawabata *et al.*, 1996).

lase. This consisted of three i.n. instillations of 10 μ l of enzyme solution in saline (split between the two nostrils) on Days 1, 3, and 10. The mice were anesthetized by inhalation of Metofane and exsanguinated on Day 15 and the sera assayed for alcalase-specific IgG1 by an ELISA. To determine the Alcalase dose response, groups of mice were treated with 0.1, 0.3, 1.0, 3.0, or 10.0 μ g of Alcalase protein per mouse. The IgG1 responses are shown in Fig. 1. The IgG1 titer increased with increasing dose. Minimal responses were seen at doses between 0.1 and 1.0 μ g/mouse. The response then increased sharply at 3.0 and 10.0 μ g/mouse. The percentage of animals responding at each dose followed a curve that closely paralleled the IgG1 titration curve.

Adjuvant Effect of a Detergent Matrix

Since exposure to enzymes in laundry detergents would occur in the presence of a detergent matrix, we looked at the effect of a detergent matrix on the IgG1 response to i.n.-dosed Alcalase. Groups of mice were treated with 0.1 to 10.0 μ g of Alcalase per mouse in the presence of 0, 10, 30, or 100 μ g of detergent. The results are shown in Fig. 2. The dose-response data in the absence of detergent matrix are the same data shown in Fig. 1. Addition of 10 μ g of detergent matrix only slightly increased the response; however, a strong adjuvant effect was seen at 30 and 100 μ g.

Figure 3 shows a comparison of the dose-response curves obtained from multiple studies in which Alcalase was administered at up to 20 μ g/mouse with or without detergent matrix. Logistics curve fits to the individual data points are plotted. The calculated ED₅₀ for enzyme dosed in detergent (0.237 μ g) is approximately fourfold less than the ED₅₀ (0.846 μ g) obtained in the absence of detergent. This demon-

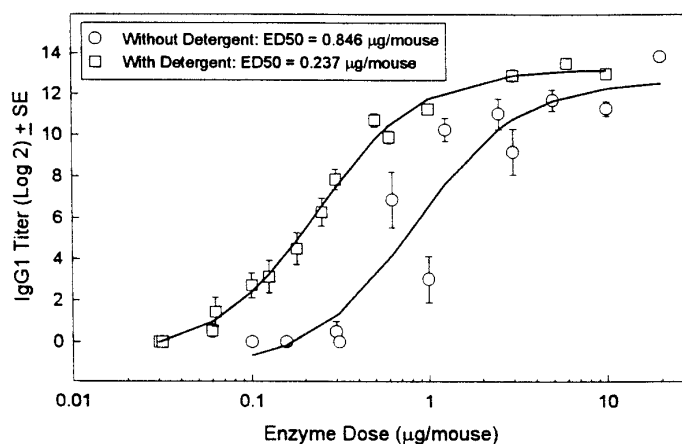


FIG. 3. BDF1 mice were administered alcalase i.n. in saline on Days 1, 3, and 10 at various concentrations with or without 30 μ g detergent. Sera were obtained on Day 15 and assayed for alcalase-specific IgG1 by ELISA (Kawabata *et al.*, 1996). The data for alcalase with detergent were compiled from seven separate dose-response studies. The data for alcalase without detergent were compiled from three separate dose-response studies. A logistics curve fit was applied to each data set and an ED₅₀ determined.

strates the strong adjuvant effect of detergent matrix in this model.

To assess the consistency of the IgG1 response to Alcalase in 30 μ g of detergent matrix, multiple studies were conducted on this enzyme over several months. The results are shown in Fig. 4. Compared to the mean ED₅₀ for six studies (0.25 μ g), the individual study ED₅₀ values were very con-

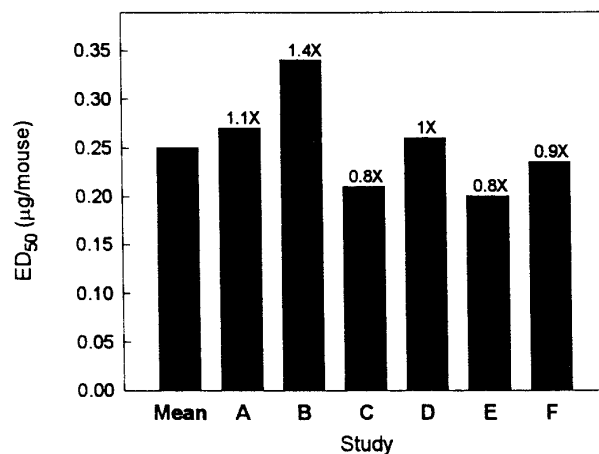


FIG. 4. Data were obtained from six independent dose-response studies of alcalase administered i.n. at various concentrations in detergent matrix to BDF1 mice. A logistics curve fit was applied to the data for each experiment and an ED₅₀ determined for each study. The mean ED₅₀ for all six studies combined and the individual study ED₅₀ values are shown. The numbers above each individual study bar indicate each study's individual ED₅₀ value divided by the mean ED₅₀ value.

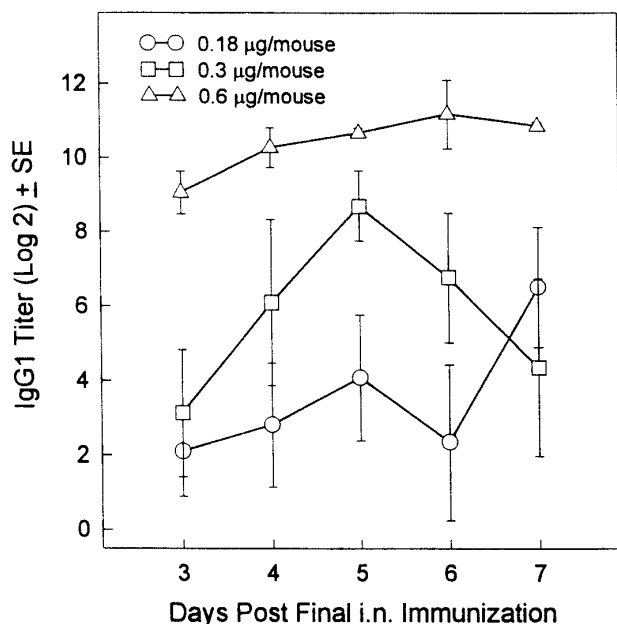


FIG. 5. BDF1 mice were administered alcalase i.n. on Days 1, 3, and 10 at the indicated concentrations in detergent matrix. Sera were obtained on the indicated days after the final administration and assayed for alcalase-specific IgG1 by ELISA (Kawabata *et al.*, 1996).

sistent, overall within 20% of the mean. We looked at both total IgG and IgG1 by ELISA to determine whether other isotypic subclasses of IgG might be induced in this model. Virtually all of the IgG response was of the IgG1 subclass (data not shown).

We also examined (on the day of bleeding—Day 15) the submandibular lymph nodes from BDF1 mice exposed i.n. to 0.25, 0.5, or 1.0 µg Alcalase in detergent matrix. Animals exposed to the higher two doses showed an approximately two-fold increase in cell counts compared to those of the mice given the lowest dose (data not shown). This may be reflective of the greater antibody response typically seen at the higher doses (Fig. 3).

Kinetics of IgG1 Response after Final i.n. Dosing

To verify the optimal timing for collection of sera, we exsanguinated groups of mice on Days 3–7 after the final i.n. dosing of Alcalase in 30 µg of detergent matrix. Different doses of Alcalase were compared to see if the optimal time would differ as a function of dose. As shown in Fig. 5, the day of serum collection was irrelevant when 0.6 µg/mouse was administered. However, when 0.3 µg was administered, the response was much more variable from animal to animal. Not all animals responded and it appeared that a Day-5 serum collection was optimal. More animals failed to show detectable IgG1 titers when the collection was delayed be-

yond 5 days. When a lower allergen dose was used (0.18 µg), the response was even more variable and an optimal serum collection time was not apparent.

Comparison of Different Mouse Strains in Their IgG1 Response to Alcalase

Early studies with the intranasal model used Balb/c (H-2^d) mice as the test strain and the response obtained was poor. Comparison studies were thus run in different inbred, outbred, and hybrid mice to identify an optimal strain. BDF1 mice (C57B1/6 × DBA/2) were initially compared with another H-2^b × H-2^d hybrid strain CB6F1 (C57B1/6 × Balb/c) as well as the outbred strains, CD1 and Swiss, and the inbred strain, Balb/c. As shown in Fig. 6, both hybrid strains responded equally well to Alcalase delivered in a detergent matrix. In contrast, the outbred CD1 and Swiss strains and the inbred Balb/c mice responded to a much lesser extent over the same dose range. Given the poor response by the Balb/c mice, it was likely that the good response by the BDF1 mice (and likewise the CB6F1 mice) was associated with the H-2^b haplotype of the C57B1/6 parent. This was confirmed by demonstrating that C57B1/6 mice showed the same dose–response profile as the F1 hybrid mouse strains. Use of the BDF1 hybrid strain for ongoing studies has resulted in a consistent and robust IgG1 response over time.

Effect of Increasing Duration of Immunization Process and Number of Doses on the IgG1 and IgE Responses to Alcalase

The ease of assay of the IgG1 response (ELISA) vs the IgE response (cellular isotope release) and the fact that IgG1

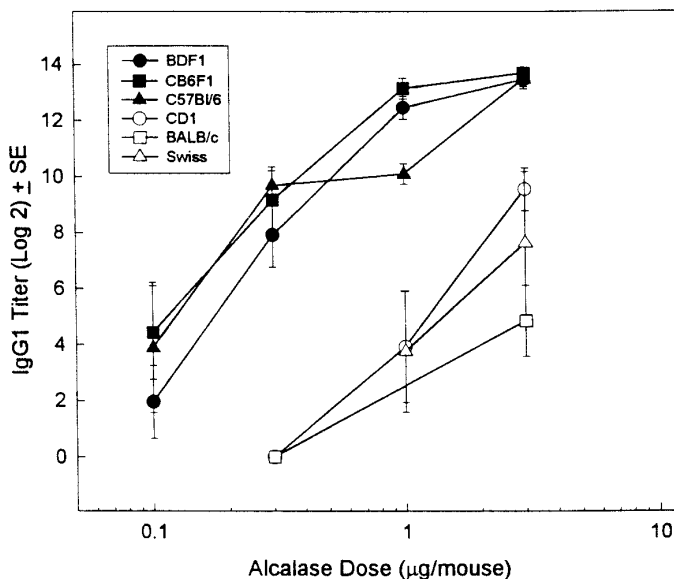


FIG. 6. Mice of the indicated strains were administered alcalase i.n. on Days 1, 3, and 10 at various concentrations in detergent matrix. Sera were obtained 5 days after the final administration and assayed for alcalase-specific IgG1 by ELISA (Kawabata *et al.*, 1996).

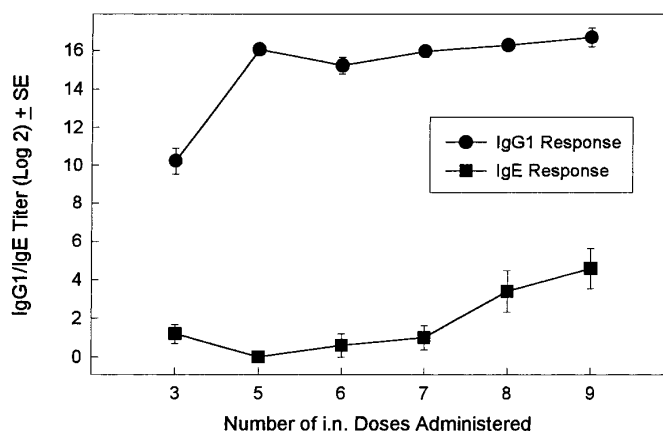


FIG. 7. One group of BDF1 mice was administered 0.5 μ g Alcalase protein (plus detergent matrix) i.n. on Days 1, 3, and 10. Other groups of mice received two to six additional doses on a weekly basis to Day 52. Sera were obtained 5 days after the last in dose for each group of mice. The sera were then assayed for alcalase-specific IgG1 by ELISA (Kawabata *et al.*, 1996) and for alcalase-specific IgE by the RBL cell [^{3} H]serotonin release assay (Kawabata and Babcock, 1993; Kawabata *et al.*, 1996).

and IgE are coregulated isotypes in mice (Purkerson and Isakson, 1992) led us to select IgG1 as a surrogate isotype for development of the i.n. model. However, since IgE is recognized as the major allergic antibody isotype in the mouse (as in man) (Revoltella and Ovary, 1969; Mancino and Ovary, 1980; Sarlo and Karol, 1994), we also assayed the serum of i.n. Alcalase-immunized mice for specific IgE using the rat basophil serotonin release assay (Kawabata and Babcock, 1993). As shown in Fig. 7, our standard three-dose immunization regimen produced a negligible IgE response. Administering up to four additional weekly doses did not improve the IgE response; however, after eight or nine weekly doses, an increase in IgE response was obtained. The increase was statistically significant after the ninth dose. The administration of five doses of Alcalase also increased the IgG1 titer, relative to the standard three dose/15 day regimen shown in Fig. 7. This IgG1 response remained at the same higher level throughout the remainder of the extended dosing regimen.

Localization of i.n. Administered Allergen

To determine the potential distribution and localization of the i.n. dosing solution throughout the respiratory tract and elsewhere, we conducted a dosing study with ^{125} I-labeled BSA. The mice were handled and positioned in the standard head down position (as used in all studies shown in Figs. 1–7) or were held in an upright position. The mice were administered 10 μ l (normal volume) or 60 μ l (exaggerated volume) split between the nostrils. The mice were exsanguinated 30 min or 2 hr after dosing and were necropsied.

Blood, trachea, esophagus, lungs, stomach, liver, kidneys and spleen, mouth, nose, head, intestines, and carcass were counted separately. The results are shown in Fig. 8.

Under our standard dosing regimen (10 μ l; head down), the majority of the dosing solution was retained in the nose after 30 min. Most of the rest was retained in the head and neck region (head, mouth, trachea). After 2 hr, a large fraction was recovered from the GI tract (stomach and intestine), though most was still retained in the nasal cavities. When a larger volume was administered (60 μ l, head down), the main difference was that a considerable fraction (>30%) was deposited in the lungs after 30 min (data not shown). When the animals were dosed with 10 μ l head up, a larger fraction (>13%) of the administered dose was deposited in the stomach after 30 min (compared to the head down position) and ~50% was deposited in the stomach after 2 hr. Less material was retained in the nasal cavities under this dosing regimen, but the amount retained in the nasal cavities was still considerable.

At later time points (data not shown), with the standard 10 μ l/down positioning, most of the radioactivity continued to be processed through the GI tract and excreted. For example, at 48 hr less than 2% of the radioactivity remained animal-associated. Of this retained radioactivity, most was in the thyroid overlying the trachea.

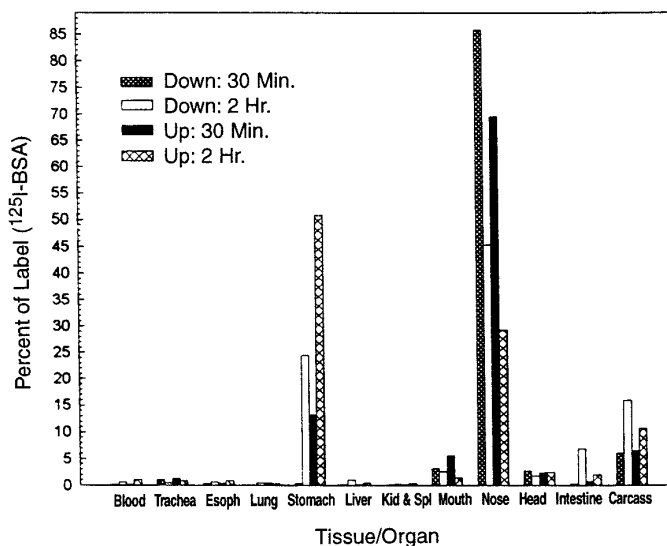


FIG. 8. BDF1 mice were administered ^{125} I-BSA in detergent matrix in 10 liters, split between the two nostrils (5 μ l per nostril). Total counts administered were approximately 1.5×10^6 . The mice were positioned in either a head up or a head down orientation. Groups of mice were euthanized after 30 min or 2 hr and the various tissues/organs collected and counted for recovered radioactivity. The percentage of label reflects the total amount of label recovered from any tissue or organ as a percentage of the total collected from all tissues/organs at each time point. The total recovered label (percentage of administered radioactivity) was approximately 85% at 30 min and approximately 84% at 2 hr.

To determine if the differences in dose distribution (between the 10 μ l head up and head down positioning) would have any impact on the biological (IgG1) response, we compared the two dosing positions in a limited Alcalase dose-response study. There was no significant difference in the magnitude of the anti-Alcalase IgG1 response when comparing head up vs head down dosing (data not shown).

Histopathology of Nasal Mucosa after Alcalase/Detergent Administration

Histopathology of the nasal cavity was done to assess any damage to the tissue as a result of the repetitive i.n. dosing procedure. In addition, we examined the tissue for evidence of acute or allergic-type inflammatory changes. In this initial study, we examined mice treated with 30 μ g detergent only, detergent plus 0.03 μ g Alcalase (a dose routinely producing no detectable IgG1 response; see Fig. 3), and detergent plus 1 μ g Alcalase (a dose producing a near maximal IgG1 response). The animals were dosed on Days 1, 3, and 10 and euthanized on Day 15. The nasal tissues were dissected and processed for microscopy.

No damage to the nasal tissue was seen in any of the animals examined. The 0 μ g Alcalase (i.e., detergent only) and low Alcalase dose groups showed unremarkable or very minimal submucosal infiltration of neutrophils along the respiratory surfaces. Animals administered 1 μ g alcalase showed only slight increases in neutrophil numbers in the submucosal tissue. No increases in neutrophils or other exudative materials were seen in the nasal lumen of any group, nor was there evidence of edema or other reactive changes in mucosal or submucosal tissues. No evidence of an allergic eosinophilic infiltration was seen in any group. Evaluation of the nasal tissues at the termination of the extended dosing study (see Fig. 7) still showed no evidence of treatment-related inflammatory changes, even after nine weekly doses of enzyme plus detergent.

DISCUSSION

The primary objective of this study was to determine the feasibility of a mouse i.n. model as an alternative to guinea pig methods (Ritz *et al.*, 1993; Santing *et al.*, 1994) or a recently developed mouse intratracheal method (Kawabata *et al.*, 1996) for the assessment of enzyme immunogenicity/allergenicity. The benchmark detergent enzyme studied, Alcalase, has had a long-standing safe history of manufacture and use as well as considerable data generated in guinea pig intratracheal and inhalation models (Ritz *et al.*, 1993). New enzymes considered for formulation into detergent products are compared to Alcalase for potency in the guinea pig test and operational exposure guidelines are set accordingly (Sarlo *et al.*, 1991). The major considerations for any alterna-

tive methodology were ease of execution, ability to quantify immunogenic/allergenic potency differences, and interstudy consistency. Prior work on development of a mouse intratracheal method (Kawabata *et al.*, 1996) fulfilled the last requirement, but not the first two.

We approached this objective by first examining various dosing regimens to identify a short-term protocol that would result in a robust response with minimal animal to animal variability. The administration of Alcalase by the i.n. route on Days 1, 3, and 10 resulted in a dose-dependent increase in Alcalase-specific IgG1 levels in serum. Two or more additional weekly doses increased the maximal specific IgG1 antibody titer to a degree. A similar dose response was observed if the data were presented as dose vs IgG1 titer or as dose vs percentage responders. However, the IgG1 titer data was preferred as it was easily converted to an ED50. The ED50 value obtained was highly consistent from study to study.

Not surprisingly, the IgG1 response to Alcalase was significantly increased (approximately fourfold) by administering the enzyme in a detergent matrix. This is consistent with results from i.t. studies in mice (Kawabata *et al.*, 1996), as well as from prior studies in guinea pigs (Markham and Wilkie, 1976, 1979) and monkeys (Cashner *et al.*, 1980). It is also consistent with numerous studies in mice showing adjuvant effects of different materials (e.g., diesel particles, pertussis antigen, cholera toxin) on antibody responses following i.n. exposures (Oien *et al.*, 1994; Tamura *et al.*, 1994b; Takafuji *et al.*, 1987; McCaskill *et al.*, 1984). The mechanism by which detergent matrix enhances the immunogenicity of an enzyme is uncertain. We are currently studying this question by looking at different detergent matrices, different enzyme/protein allergens, and multiple endpoints (antibody response, cellular immunoinflammatory response, histopathology, etc.). Since detergent manufacturing plant exposure to enzymes occurs in the presence of detergent matrix, the comparative potency determinations between Alcalase and other enzymes of interest will generally be conducted with preparations containing detergent (Ritz *et al.*, 1993; Kawabata *et al.*, 1995b; Sarlo *et al.*, 1991).

Immunogenetic diversity in murine responsiveness to protein allergens has been recognized for many years. Different inbred strains of mice will respond differently depending on the immunogen, the adjuvant, the antibody isotype of interest, and the route of administration (Tomlinson *et al.*, 1989; Else and Wakelin, 1989; Gurvich and Korukova, 1986; Nakashima *et al.*, 1983; Revoltella and Ovary, 1969; Mancino and Ovary, 1980; Staruch and Wood, 1982; Kudo *et al.*, 1978; Petty and Steward, 1977; Berzofsky *et al.*, 1977; Fujiwara *et al.*, 1976; Nomoto *et al.*, 1972). Though Balb/c mice have been good responders in other systems, including the i.t. model (Tomlinson *et al.*, 1989; Gurvich and Korukova,

1986; Mancino and Ovary, 1980; Staruch and Wood, 1982; Kudo *et al.*, 1978; Kawabata *et al.*, 1996), they responded poorly in the i.n. model. Initial testing of hybrid (H-2^b × H-2^d) mice suggested that the high responder phenotype might be H-2^b associated. This was confirmed by the high response demonstrated by the inbred C57Bl/6 (H-2^b) mice.

In contrast to the robust and consistent IgG1 response, the basic i.n. dosing regimen used for Alcalase produced only a weak IgE response. This finding was in contrast to the results of the i.t. model in which both a strong IgG1 response and consistent, albeit lower, IgE response were detectable (Kawabata *et al.*, 1996). Administration of antigen by the i.n. route has been shown to induce IgE responses in a variety of systems (Holt *et al.*, 1981a; McCaskill *et al.*, 1982, 1984; Enander *et al.*, 1985; Henderson *et al.*, 1985, 1987; Takafuji *et al.*, 1987, 1989; Tamura *et al.*, 1994a). The magnitude of the IgE response varies from system to system and is sometimes dependent on adjuvant administration. In our system, the IgE response was evident after extending the dosing regimen several weeks. This is consistent with findings from the i.t. model in which a 4-week regimen was required to observe an IgE response and 6 weeks was required to maximize the response (Kawabata *et al.*, 1996).

The difference between i.t. and i.n. dosing, in terms of the delay and lower magnitude of the IgE response in the i.n. model, may reflect the minimal distribution of enzyme to the lungs after i.n. dosing, as suggested by our results with ¹²⁵I-labeled BSA. Even though mucosal antigen-presenting Langerhans cells line the entire respiratory tract, including the nasal regions (Holt *et al.*, 1988, 1989; Fokkens *et al.*, 1989), distribution of the antigen throughout the respiratory tract may be needed for triggering rapid and optimal IgE responsiveness. Further studies are needed with actual protease enzymes to further assess the validity of the BSA results.

The use of IgG1 as a surrogate endpoint for IgE in this model is open to criticism for trying to predict relative allergenicity of enzymes without relying on the major allergenic antibody isotype. However, there are several reasons we believe it is appropriate to do so. First, IgG1 is coregulated with IgE via the IL-4/TH2 pathway (Purkerson and Isakson, 1992). Second, there is some evidence to suggest that allergic responses can occur in the absence of detectable IgE responses (Oettgen *et al.*, 1994; Lei *et al.*, 1996). Third, a recent study (Oshiba *et al.*, 1996) showed that passive transfer of *either* ovalbumin-specific IgE or IgG1 antibody into naive mice produced, to a similar degree, ovalbumin-specific immediate skin test reactivity and, upon inhalation challenge, respiratory hyperresponsiveness. IgG2a and IgG3 isotypes were not able to transfer responsiveness. Last, we have preliminary evidence that the mouse i.n./IgG1 method produces relative potency data similar to that obtained in prior GPIT testing. Our testing of multiple enzymes of several

classes (proteases, amylases, cellulases, lipases) has shown similarity in potency differences when compared to prior GPIT studies (Horn *et al.*, 1996, Kimber *et al.*, 1996, and manuscript in preparation). Thus, we believe that the IgG1 response endpoint will be suitable for comparative immunogenicity assessments of enzymes. However, given the current lack of evidence of allergic response (IgE, histopathology, respiratory signs) we are continuing to investigate the effect of alternative dosing regimens in the i.n. model in our efforts to further develop and characterize the method as an allergenicity model.

Nasal histopathology failed to show evidence of tissue-destructive changes or inflammation due to the repetitive dosing procedure and resulting sensitization process. The reason for this lack of inflammatory response indicative of allergy (e.g., tissue eosinophilia) (Kung *et al.*, 1994a) was not clear and is currently being reexamined. If alternative i.n. dosing regimens eventually lead to a strong IgE response, it is possible that evidence of tissue eosinophilia and/or activated Th2 cells would become manifest (Lukacs *et al.*, 1994; Kung *et al.*, 1994a,b; Garlisi *et al.*, 1995; Bursselle *et al.*, 1995).

It is not necessary that disease manifestations of allergic inflammation occur in this model to make it valid for the purpose of assessing relative enzyme immunogenicity/allergenicity. Many newly emerging methods in immunotoxicology rely on surrogate endpoints or endpoints that measure only a component of the response process (e.g., skin equivalent cultures and measure of viability or IL-1 cytokine release for skin and eye irritation, local lymph node assay for contact sensitization, mouse IgE test for chemical respiratory sensitization). Their value lies in their predictive capability, not in their precise pathophysiological or mechanistic adherence to the clinical disease or response process. We acknowledge that evidence of nasal tissue eosinophilia, activated T cell infiltration, and/or respiratory signs would add support to the contention that i.n. dosing of enzymes can lead to allergic manifestations. We are examining this question in ongoing studies to further characterize the model.

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