

Use of the Mouse Intranasal Test (MINT) to Determine the Allergenic Potency of Detergent Enzymes: Comparison to the Guinea Pig Intratracheal (GPIT) Test¹

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A mouse intranasal test (MINT) was developed to determine the relative allergenicity of detergent enzymes. In this simple method, various doses of the enzymes are administered in a detergent matrix, via intranasal instillation, on days 1, 3, and 10, with serum samples collected on day 15. The sera are then analyzed for enzyme specific IgG1 antibody by an antigen specific enzyme immunoassay. The protease enzyme Alcalase (protease Subtilisin Carlsberg) has been used as a benchmark enzyme for development and characterization of the model. The objective of the current studies was to obtain potency comparisons with other protease and nonprotease enzymes and to begin to assess the validity of the model by comparison with potency determinations obtained with the guinea pig intratracheal (GPIT) test. The range of potencies detected among several enzymes of different classes was approximately 60-fold (compared with Alcalase). Modification of the dosing regimen to permit slightly more extended dosing did not change the relative potency determination. Comparison of data from the MINT and GPIT methods indicate that both the mouse and the guinea pig recognize the bacterial amylase Termamyl and a fungal exocellulase as more potent than Alcalase, a serine protease (Subtilisin B) and a fungal α -amylase (Fungamyl) as less potent than Alcalase, and the serine protease, Savinase, as equivalent to Alcalase. Also, these data are in alignment with clinical data on the prevalence of occupational enzyme sensitization. Given the simplicity and low cost of the MINT method compared with the GPIT test, these results support continued development of the method as an alternative approach for assessing the allergenicity of enzymes. © 1998 Society of Toxicology.

Enzymes have been shown to produce type I respiratory allergy in workers at laundry detergent manufacturing plants

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(Flindt, 1969; Pepys *et al.*, 1969). However, the application of threshold limit values (TLV)³ and operational exposure guidelines (OEG) along with the stringent control of enzyme dust levels in plants has minimized the incidence rate of sensitization (Juniper *et al.*, 1977). This safe level was empirically established for the protease, Subtilisin Carlsberg (Alcalase). As new enzymes are introduced into detergent plants, there is a need to evaluate their allergenicity compared with Alcalase, as a benchmark, and to determine the correct TLVs and OEGs for each enzyme.

To address this need, the guinea pig intratracheal (GPIT) test was developed (Ritz *et al.*, 1993). In this model, guinea pigs were intratracheally (i.t.) administered the test enzyme in detergent matrix and the enzyme specific IgG1 antibody response was measured as the index of sensitization (IgG1 is the major anaphylactic antibody in the guinea pig). The relative potencies of different enzymes of the protease class, as well as nonprotease enzymes, were determined using the GPIT method (Sarlo *et al.*, 1997a,b). Using the relative potencies determined via the GPIT, OEGs were established for detergent manufacturing plants. Medical surveillance in these plants has verified the predictive utility of the GPIT. Although the lack of immunobiological reagents specific for the guinea pig has hampered somewhat the ability to conduct mechanistic studies, the GPIT method has been utilized to study certain pathophysiological aspects of the enzyme sensitization process (Fletcher and Sarlo, 1997; Sarlo *et al.*, 1997b). The major limitations of the GPIT are that it is time consuming and expensive. This limits its application as a screening tool.

To address these needs, we have worked to develop alternative mouse models to assess enzyme allergenicity. A first model, an i.t. dosing model (Kawabata *et al.*, 1996), was developed to incorporate a dosing method similar to the GPIT and proved useful but also technically difficult. More recently

³ Abbreviations used: DPBS, Dulbecco's phosphate-buffered saline; ED50, effective dose for 50% of maximum response; ELISA, enzyme-linked immunosorbent assay; GPIT, guinea pig intratracheal; i.n., intranasal; i.t., intratracheal; MINT, mouse intranasal test; OD, optical density; OEG, operational exposure guideline; PCA, passive cutaneous anaphylaxis; TLV, threshold limit value.

a mouse intranasal test (MINT) method was developed (Robinson *et al.*, 1996; Robinson and Kawabata, 1997). This model requires relatively short-term dosing compared with either the GPIT or mouse i.t. tests and is therefore both cost and time effective. This model demonstrates reproducible potency determinations for Alcalase and shows detergent adjuvant effects similar to that seen with the GPIT, mouse i.t., and other animal model systems (Cashner *et al.*, 1980; Kawabata *et al.*, 1996; Markham and Wilkie, 1976, 1979). The MINT model relies on antigen specific IgG1 as the index of allergenic potency. The dosing regimen has to be extended in order to detect antigen specific IgE responses (Robinson *et al.*, 1996). However, the IgG1 response has been used as an allergenicity endpoint based on the fact that it is regulated via the TH2/IL-4 pathway (as is IgE) (Purkerson and Isakson, 1992) and evidence from multiple laboratories that IgG1 is also a homocytotropic antibody isotype that can drive the type I allergic/anaphylactic response in mice (Facincone *et al.*, 1997; Hamelmann *et al.*, 1997; Lei *et al.*, 1996; Mehlhop *et al.*, 1997; Miyajima *et al.*, 1997; Nussensweig *et al.*, 1964; Oettgen *et al.*, 1994; Oshiba *et al.*, 1996).

The purpose of the present studies was to assess the relative potencies of detergent enzymes (protease and nonprotease) using the MINT method. Enzyme potencies greater than, equal to, and less than the Alcalase benchmark were identified. Most important, the potency determinations made via the MINT method were very similar to those obtained previously using the GPIT test. Evaluation of occupationally exposed workers showed that the development of clinical sensitization to enzymes (defined as skin prick test conversions) followed what would be predicted from the guinea pig model (Sarlo *et al.*, 1997a). Therefore, a good correlation between the results generated in the MINT and those from GPIT test strongly support continued development and validation of the mouse model as a means of determining the allergenic potencies of enzymes. It may also be possible to extend the method to assessment of other nonenzyme proteins of commercial or medical value, such as food allergens or therapeutic proteins or peptides.

MATERIALS AND METHODS

Animals. Female BDF1 mice (Charles River Laboratories, Portage, MI) weighing 18–20 g were used in all intranasal studies. The mice were housed in cages with wood chip bedding. Female Hartley guinea pigs (Charles River Laboratories) weighing 300–400 g were used in the intratracheal studies and were individually housed in stainless steel, wire-bottom cages. All animals were quarantined 2 weeks prior to placement in the studies. Guinea pigs and mice were randomized by body weight into equal sized dose groups. All of the animals were kept in rooms controlled for humidity (30–70%), temperature (67–77°F), and 12-h light and dark cycles. Animals were allowed feed (Purina mouse or guinea pig chow, Purina Mills, Richmond, IN) and water *ad libitum*. All animal treatments were done in accordance with procedures approved by an Institutional Animal Care and Use Committee. The NIH guidelines for the care and use of laboratory animals were followed throughout the course of these studies. Ten guinea pigs and five mice were included in each dosing group across all studies.

Test chemicals. All test detergent enzymes were of bacterial or fungal origin. Alcalase (serine protease), Savinase (serine protease), Termamyl (bacterial amylase), Fungamyl (fungal α -amylase), and exocellulase were obtained from Novo Industri A/S (Bagsvaerd, Denmark). Subtilisin B (serine protease) was obtained from Genencor International (South San Francisco, CA). Protein levels were determined by Kjeldahl nitrogen analysis and were as follows: Alcalase [35.4%], Savinase [21.2%], Termamyl [3 different lots; 4.6, 22.2, and 52%], Fungamyl [10.4%], exocellulase [6.3%], and Subtilisin B [4.4%]. Other material in enzyme receipts (e.g., inorganic ash, carbohydrate) varied in percentage composition. However, most of the total protein nitrogen in each enzyme receipt was active enzyme. Regardless, all enzymes were dosed on an equivalent protein weight basis in 0.9% sodium chloride containing 3 mg/ml of a detergent matrix for the mouse studies or 2.5 mg/ml of a detergent matrix for guinea pig studies. The detergent matrix used consisted primarily of anionic and nonionic surfactants, silicate builders, and perborate bleach and was formulated by the Procter & Gamble Company.

Intranasal dosing method. Mice were anesthetized by an intraperitoneal (i.p.) injection of a mixture of Ketaset (88.8 mg/kg) and Rompun (6.67 mg/kg). The anesthetized animals were held in the palm of the hand, backs down, and dosed intranasally with enzyme in saline plus detergent matrix. Dosing solutions were administered in volumes of 10 μ l/mouse divided equally between the two nostrils. 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 their backs upon a mound of bedding with their heads angled slightly downward. This orientation slightly prolongs the retention of the dosing solution in the nasal cavity (Robinson *et al.*, 1996). Dosing was repeated on days 1, 3, and 10 and blood was collected on day 15, unless otherwise indicated.

Guinea pig intratracheal dosing method. Restrained, unanesthetized guinea pigs were dosed by the intratracheal route with 0.1 ml of enzyme protein in saline + detergent solution once per week for 10 consecutive weeks as previously described (Ritz *et al.*, 1993). All enzymes were dosed on an equivalent protein basis and the doses were delivered near the tracheal bifurcation by inserting a 2-inch, 18-gauge ball-tipped gavage needle into the tracheas and injecting the dose during inspiration. Immediately after being dosed, each animal was observed for signs of respiratory distress as demonstrated by periodic diaphragmatic spasms or retractions. Doses of enzyme were chosen to minimize the severity of the respiratory response (no anaphylaxis) and to maintain the group size throughout the course of the study.

Enzyme linked immunosorbent assay (ELISA) for mouse IgG1 antibody. Enzyme-specific IgG1 in mouse serum was, for most studies, measured by an antigen capture ELISA method as previously described (Kawabata *et al.*, 1996; Robinson *et al.*, 1996). For each dilution of serum tested, the optical density (OD) of normal sera tested 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 (Softmax, Molecular Devices, Menlo Park, CA). A linear portion of each curve was derived and the serum dilution at an absorbance of 0.5 OD was calculated by interpolation. Titers for serum samples were expressed as log 2 of the reciprocal of the interpolated dilution (i.e., 2 to 14 representing 1:4 to 1:16,384 dilution range).

In certain studies (identified in figure legends), an antibody capture procedure was used. In this procedure, the test wells were first incubated overnight in rabbit anti-enzyme antibody (produced in-house, via contract, or obtained from the enzyme supplier) at 4°C. The proper dilution of antibody to use was determined through an assay optimization procedure, but was in the range of 1/500 to 1/2000. The antibody binding step was followed by a 1-h incubation of each well with 200 μ l of Dulbecco's phosphate buffered saline (DPBS, Sigma Chemical Co., St. Louis, MO) containing 0.05% Tween 20 as a wash buffer. This was both a wash step and a blocking step to prevent nonspecific binding of protein to the wells. Next the appropriate antigen (enzyme) was added at a predetermined optimal dilution to each well in buffer containing 1% bovine serum albumin (Sigma) and incubated for 1 h at 37°C. The wells were then washed and further incubated in serial dilutions of test sera followed by alkaline phosphatase-conjugated anti-mouse IgG1 antibody (Southern Biotech-

nology Associates, Inc., Birmingham, AL) and substrate (*p*-nitrophenylphosphate, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) as described for the antigen capture procedure (Robinson *et al.*, 1996). The titer determination procedure for the antibody capture ELISA was the same as that described above.

Passive cutaneous anaphylaxis (PCA) assay for guinea pig homocytotropic antibody. Circulating IgG1 homocytotropic antibodies in sera from i.t. exposed guinea pigs were measured in a 4-h PCA test as previously described (Ritz *et al.*, 1993). Naive guinea pigs received intradermal injections of sera diluted 1:4 to 1:16,384 in physiological saline. Normal serum (1:4) and saline served as controls. Four hours later, the animals were lightly ether-anesthetized and injected intracardially with 2 ml/kg body weight of a physiological saline solution containing 1% Evans blue dye (Sigma, St. Louis, MO) and 100 μ g protein/ml of the appropriate enzyme. The highest dilution of serum showing a positive response (any visible bluing at the intradermal injection site) was the end titer. The PCA antibody titer was expressed as the logarithm to the base 2 of the reciprocal of the end point dilution as described above.

Data analysis. In the mouse studies, ED₅₀ values (the dose of enzyme producing a half maximal serum IgG1 titer) were determined from the IgG1 response versus enzyme dose curve. These were determined using the SigmaPlot logistics curve fitting software program (SigmaPlot for Windows, Version 2.0, Jandel Scientific, San Rafael, CA). Relative enzyme potency determinations were made by dividing the ED₅₀ value obtained for Alcalase by the ED₅₀ value obtained for the test enzyme. In the guinea pig studies, estimation of potency differences was made as previously described (Sarlo *et al.*, 1997a). Briefly, comparisons between Alcalase and test enzymes were made during the early part of the study (i.e., weeks 4, 5, and 6) when the differences in antibody titer were most apparent. Test enzymes were considered more or less potent than Alcalase if significant differences ($p < 0.05$) between antibody titers were observed at more than one dose level and more than one time point. A test enzyme was considered more potent than Alcalase if the dose-response over time curves were shifted to the left of the curves for Alcalase. A less potent enzyme would generate curves shifted to the right of the Alcalase curves.

RESULTS

Relative potency of Alcalase and other protease enzymes. Alcalase and the protease enzymes, Subtilisin B and Savinase, were administered on an equivalent protein weight basis in detergent matrix to separate groups of BDF1 mice. Alcalase was included in the separate studies for direct comparison to the other proteases. The dose-response curves for the two protease enzymes are shown in Fig. 1. Subtilisin B was approximately half as potent as Alcalase based on the comparative ED₅₀ values (Fig. 1A). Savinase showed approximately equal potency (Fig. 1B).

Relative potency of Alcalase and other nonprotease enzymes. Similar to the protease studies, dose-response comparisons of Alcalase and the nonprotease enzymes, Termamyl Fungamyl, and an exocellulase were conducted. Figure 2 shows the results of three separate studies with Termamyl conducted over a 1-year period. Figures 2A and 2B show independent comparison studies of the same lot of Termamyl in direct comparison with Alcalase. The two studies showed similar potency differentials (4.8 $\times$ and 3.5 $\times$), with Termamyl having the greater potency in both studies. In a third study, three different lots of Termamyl with widely varying protein contents were compared. In each group of test animals, the

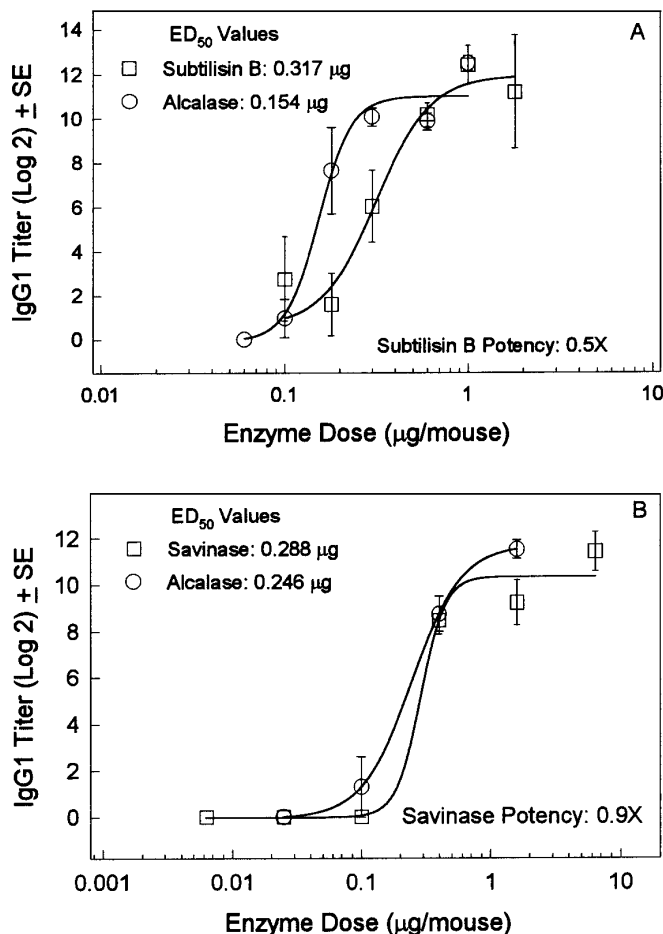


FIG. 1. BDF1 mice were administered i.n. Alcalase or test enzyme (Subtilisin B (A) or Savinase (B) in detergent matrix on days 1, 3, and 10 at the indicated doses. Sera were obtained on day 15 and assayed for enzyme specific IgG1 by ELISA.

enzymes were administered on an equal protein weight basis. In conducting this comparison study, the enzyme immunoassay was modified to an antibody capture procedure in which a common rabbit anti-Termamyl antibody was initially used to coat the immunoassay plates. Figure 2C shows the results of this comparison. The three Termamyl enzyme lots had similar ED₅₀ values (0.056–0.087 μ g/mouse) which, in comparison to the Alcalase benchmark, resulted in similar relative potencies (3.9 $\times$ –6.0 $\times$) that were also in line with earlier studies (Figs. 2A and 2B), indicating that enzyme potency was not dramatically altered by differences in enzyme purity.

Though Fungamyl is a member of the same general enzyme class as Termamyl (amylase), it is of fungal versus bacterial origin. It also showed a very different potency response profile. The MINT results are shown in Fig. 3. The threshold dose for a very low level response to Fungamyl (i.e., single animal response) was similar to that of Alcalase; however, the full dose-response pattern was very different in that it took a dose of >10 μ g to maximize the IgG1 response. Dose levels that produced a maxi-

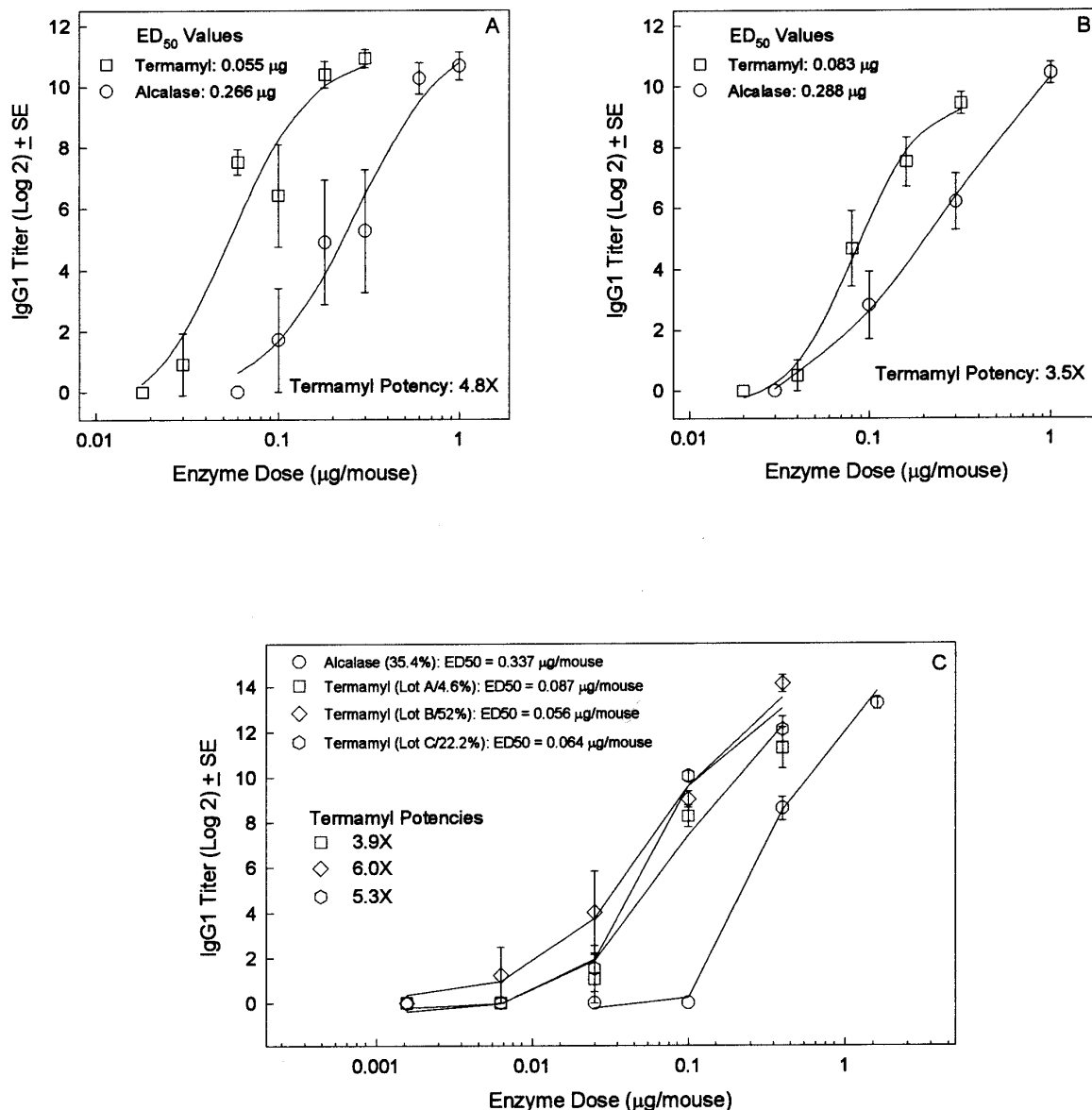


FIG. 2. BDF1 mice were administered i.n. Alcalase or Termamyl in detergent matrix on days 1, 3, and 10 at the indicated doses. Sera were obtained on day 15 and assayed for enzyme specific IgG1 by ELISA. (A–C) Three separate experiments. In one experiment (C) three different lots of Termamyl (with varying levels of enzyme protein) were compared for potency. An antibody capture ELISA method was used for this experiment (see Materials and Methods).

mal anti-Alcalase IgG1 response, produced only a half maximal anti-Fungamyl response. This resulted in a differential potency of approximately 10 $\times$ with Fungamyl calculated as being only 0.094 $\times$ as potent as Alcalase based on these results.

We also tested a representative enzyme of the cellulase class, a fungal exocellulase, with the response pattern and potency determination shown in Fig. 4. The exocellulase enzyme had a dose–response curve clearly shifted to the left relative to Alcalase, particularly at the low and intermediate doses administered. This resulted in a relative potency calculation of 3.4 $\times$.

Effect of dosing regimen on relative enzyme potency determinations. The GPIT evaluates enzyme allergenicity over time at several administered doses (Sarlo *et al.*, 1997a). In contrast, the MINT takes only a “snapshot” of the dose response at a single point in time. It was shown previously that the mouse IgG1 response to Alcalase will increase 4–6 log 2 units (16–64 $\times$) when the dosing regimen is extended from three doses over 10 days to five doses over 24 days (Robinson *et al.*, 1996). Additional weekly doses (up to nine doses over 52 days) did not further increase the IgG1 titers obtained. The objective of this study was to determine if a potency determi-

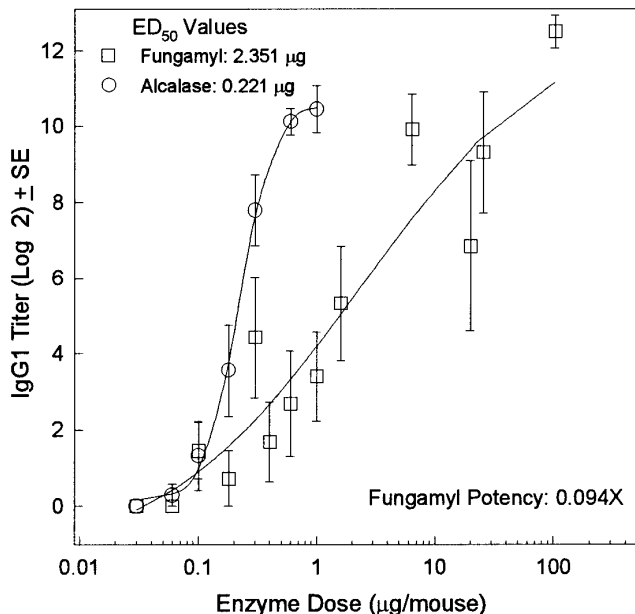


FIG. 3. BDF1 mice were administered i.n. Alcalase or Fungamyl in detergent matrix on days 1, 3, and 10 at the indicated doses. Sera were obtained on day 15 and assayed for enzyme specific IgG1 by ELISA.

nation obtained using the “standard” regimen would be altered under a different dosing regimen. Termamyl was used as the test enzyme due to its consistently greater potency than Alcalase in repeat standard dosing studies (Fig. 2).

In the study shown in Fig. 5, different animal groups were

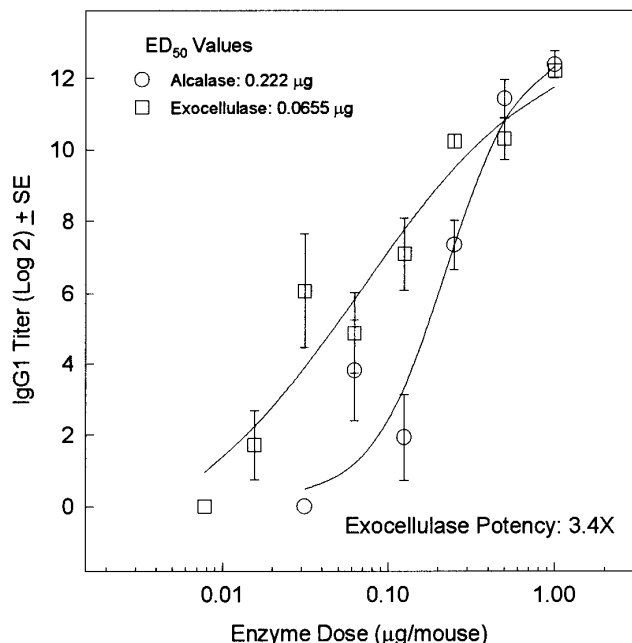


FIG. 4. BDF1 mice were administered i.n. Alcalase or exocellulase in detergent matrix on days 1, 3, and 10 at the indicated doses. Sera were obtained on day 15 and assayed for enzyme specific IgG1 by ELISA.

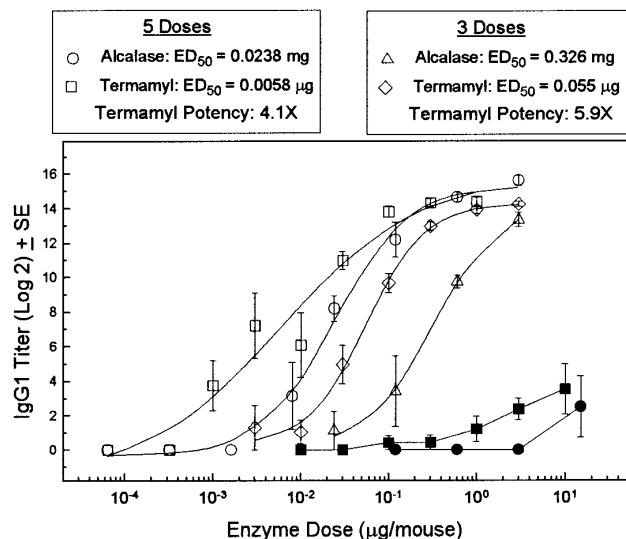


FIG. 5. BDF1 mice were administered i.n. Alcalase or Termamyl in detergent matrix on days 1 and 3; days 1, 3, and 10; or days 1, 3, 10, 17, and 24 at the indicated doses. Sera were obtained 5 days after the last intranasal dose and assayed for enzyme specific IgG1 by ELISA. ED₅₀ values were not determined for animals dosed only twice with Alcalase (●) or Termamyl (■) since incomplete dose-response curves were obtained.

administered two, three, or five doses of Alcalase or Termamyl over 3, 10, or 24 days, respectively, with serum obtained 5 days after the last intranasal enzyme dose. The dose-response curves shown in the figure indicate that Termamyl was the more potent enzyme regardless of the dosing regimen used. At fewer doses administered, larger concentrations of enzyme had to be given to produce the same antibody titer, thus the ED₅₀ values changed dramatically between the five-dose and the three-dose regimens. However, the relative enzyme potencies based on these ED₅₀ values were very similar and in line with those of our earlier studies (Fig. 2). The two-dose regimen did not produce high enough IgG1 titers to enable us to develop a full dose-response curve. However, even with two doses, a separation in dose-response curves was beginning to appear at the high enzyme levels, again with anti-Termamyl IgG1 titers appearing at the lower administered doses.

Relative enzyme potency determinations: GPIT vs MINT. All of the enzymes tested in the MINT model were previously tested in the GPIT. Some of these data (comparative results for Alcalase, Savinase, Subtilisin B, and Termamyl) have been published (Sarlo *et al.*, 1997a). The comparative GPIT data for Fungamyl and exocellulase are presented in Fig. 6. In the case of Fungamyl (Fig. 6A), the response at the two lowest doses was negative. The time course response to 3.0 μg Fungamyl was approximately the same at that obtained with 0.3 μg Alcalase. Hence, the relative potency for Fungamyl was estimated as 0.1X. For the exocellulase/Alcalase comparison (Fig. 6B), the meaningful comparative data were seen at weeks 4–6, during the period of increasing titers when differences in

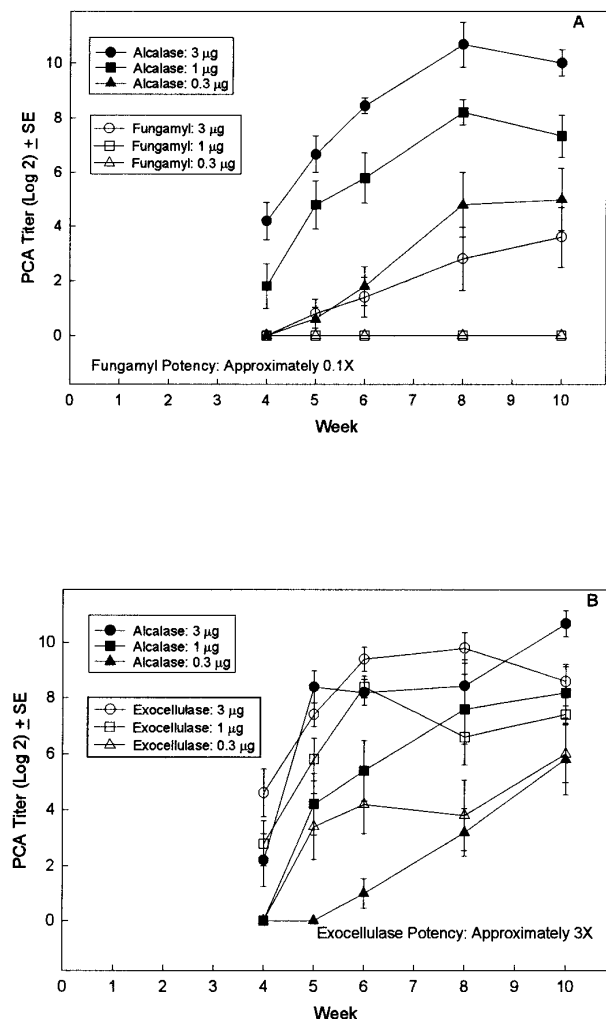


FIG. 6. (A) Allergic antibody titers in sera from guinea pigs dosed with Alcalase or Fungamyl. The titers from animals exposed to 3.0 μ g Fungamyl were not different from the titers from animals exposed to 0.3 μ g Alcalase. (B) Allergic antibody titers in sera from guinea pigs dosed with Alcalase or exocellulase. The titers from animals exposed to 0.3 μ g exocellulase were not different from the titers from animals exposed to 1.0 μ g Alcalase at weeks 4 through 6. The titers from animals exposed to 1.0 μ g exocellulase were not different from the titers from animals exposed to 3.0 μ g Alcalase at weeks 4 and 6.

antibody titers are most apparent (Sarlo *et al.*, 1997a). At only one dose level and time point during this interval (3 μ g/5 weeks) did the two enzymes show equal potency; otherwise, the 0.3- μ g dose of exocellulase produced a response similar to that seen with 1.0 μ g of Alcalase and the 1.0- μ g dose of exocellulase produced a response similar to that of the 3.0- μ g dose of Alcalase. On this basis, the relative potency for exocellulase was determined to be approximately 3 $\times$ that of Alcalase.

The relative (to Alcalase) potency results for all the protease and non protease enzymes described in this paper are compared in Table 1 to the prior data obtained in the GPIT. Overall, with the enzymes tested to date, there has been a

strong correspondence in the relative enzyme potencies determined by these two methods. The various potency differentials detected by the MINT in Table 1 indicate that the model is able to determine potency differences over a range of at least 60 $\times$ relative to Alcalase (i.e., 10-fold less potent to 6-fold more potent).

DISCUSSION

The primary objective of this study was to determine the feasibility of a MINT model (Robinson *et al.*, 1996) as an alternative to the GPIT test (Ritz *et al.*, 1993; Sarlo *et al.*, 1997a) for the assessment of enzyme allergenicity. The benchmark detergent enzyme 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 with Alcalase for potency in the GPIT test and operational exposure guidelines are set accordingly (Sarlo *et al.*, 1997a). The relative prevalence of new sensitizations to these enzymes in the manufacturing facilities has supported the validity of the GPIT for enzyme allergenicity potency assessment. The guinea pig has been widely used in relatively short-term studies as an experimental system for modeling asthma pathophysiology and drug therapeutics (Kudlacz *et al.*, 1996; Sagara *et al.*, 1997). However, the GPIT is a more chronic dosing model than those typically employed for asthma studies. Hence, the major drawback of the GPIT has been its lengthy study methodology and the resultant high cost. The GPIT method has been successfully used to study critical aspects of the pathogenesis of enzyme allergenicity, such as the enhancing effects of protease on nonprotease enzymes (Sarlo *et al.*, 1997b) and the effects of peak versus low level enzyme exposures in triggering sensitization (Fletcher and Sarlo, 1997). Still, the complexity of the model has limited its application as a screening tool.

TABLE 1
Comparison of Relative Enzyme Potencies (vs Alcalase) Based on Guinea Pig Intratracheal (GPIT) and Mouse Intranasal (MINT) Testing

Enzyme	Enzyme potency vs Alcalase	
	GPIT potency vs Alcalase ^a	MINT potency vs Alcalase ^b
Savinase	Approximately 1 $\times$ as potent	0.9 $\times$
Subtilisin B	0.3 $\times$ as potent	0.5 $\times$
Termamyl	Approximately 10 $\times$ more potent	3.5–6.0 $\times$
Fungamyl	0.1 $\times$ as potent	0.094 $\times$
Exocellulase	Approximately 3 $\times$ more potent	3.4 $\times$

^a Potencies from past GPIT tests as published (Sarlo *et al.*, 1997a) or as presented in Fig. 6.

^b Potencies based on differential ED₅₀ values for Alcalase and the test enzymes as shown in Figs. 1–4.

The MINT was developed as a rapid and low cost alternative to the GPIT test (Robinson *et al.*, 1996). The focus of the studies presented here was to verify the utility of the model based on initial determination of relative enzyme allergenic potencies in comparison to past results obtained in the GPIT. As previously reported, the MINT relies on assessment of enzyme specific IgG1 antibody as an index of allergenicity (Robinson *et al.*, 1996). Enzyme specific IgE can be detected in the MINT, but requires longer term dosing and uses a more difficult and less quantitative assay. However, IgG1 is considered a reasonable surrogate endpoint because of its common regulation with IgE via the TH2/IL-4/IL-5 pathways (Purkerson and Isakson, 1992). While it has been known for many years that IgG1 is also a homocytotropic isotype in mice (Nussensweig *et al.*, 1964), it has recently also become well established that IgG1 is an important mediator of type I hypersensitivity in the mouse (Facincone *et al.*, 1997; Hamelmann *et al.*, 1997; Lei *et al.*, 1996; Mehlhop *et al.*, 1997; Miyajima *et al.*, 1997; Oettgen *et al.*, 1994; Oshiba *et al.*, 1996) and thus can be used as a true indicator of allergic sensitization. Interestingly, the analogous isotype in humans (IgG4) has also recently been associated with type I hypersensitivity to protein allergens (Awazuhara *et al.*, 1997; Codina *et al.*, 1997; Schuyler *et al.*, 1997), including the induction of sensitization via the respiratory tract in previously naive individuals (Schuyler *et al.*, 1997).

The potential value of the MINT as an alternate method to the GPIT to assess relative enzyme allergenicity requires that similar profiles of relative enzyme potency be obtained since a database accumulated over many years has documented the utility of the GPIT in predicting allergenic potency in humans. For example, the prevalence of sensitization to specific enzymes in detergent manufacturing plants has been determined via skin prick test conversion from negative to positive with or without symptoms (Sarlo *et al.*, 1997a). Enzyme exposure levels are established in these detergent plants by the relative enzyme allergenic potencies determined by the GPIT test. Thus, if an enzyme is 3 $\times$ more potent than Alcalase in the GPIT test, its exposure guideline is set at one-third that of Alcalase. If the potency determinations are correct, this practice would equalize the relative prevalence of sensitization (prick test conversions). Enzymes that are equal or less potent than Alcalase are held at the Alcalase exposure guideline. Therefore, equipotent enzymes would be expected to show equal (and less potent enzymes show lower) prevalences of sensitization.

The good correlation between the prevalence of occupational sensitization for several enzymes and their relative potencies in the GPIT has recently been published (Sarlo *et al.*, 1997a). Based on the results reported in Figs. 1–4 and Table 1, the MINT method, though taking less than 25% as long as the GPIT test and costing only one-fifth as much, has thus far largely confirmed all of the previous GPIT test results. For each enzyme tested (Savinase, Termamyl, Subtilisin B, Fungamyl, and the exocellulase), good agreement was obtained on relative potency (vs Alcalase)

between the mouse and the guinea pig models. The relative enzyme potency determinations were also consistent for different lots of enzyme with widely varying degrees of purity, not unexpected since the enzymes were administered on a consistent enzyme protein weight basis. Given that the GPIT test is a protracted dosing regimen, and that earlier studies had shown an increase in IgG1 titers after extended enzyme dosing in mice, it was important to determine if the potency differences seen in the mouse were highly restricted to a single specific dosing regimen. The results in Fig. 5 showed that this does not appear to be the case. Administering fewer or more doses than the standard three-dose regimen drastically shifted the dose–response curves, but the relative potencies of the two enzymes were maintained. Last, although independently determined dose–response curves for Alcalase were fairly consistent from study to study, a 10–20% variation around a mean ED50 of approximately 0.25 μ g/mouse (Robinson *et al.*, 1996) was found. Thus, it is important to include Alcalase as a positive control in each study.

Though various statistical methods could be used to compare enzyme potencies from dose–response curves, the use of relative ED50 values is a standard pharmacologic tool that has been applied to this method since the midpoint of the dose–response curve can be defined regardless of the shape of the curve. In terms of potency comparisons the lack of parallelism in dose–response curves can be confounding. This is seen in the Alcalase/Fungamyl comparison (Fig. 3), where an ED10 or ED80 comparison would have given very different relative potency calculations than the ED50. From a practical standpoint, it is difficult to use an ED10 (or threshold) comparison since the response in each animal tends to be “all or nothing,” with very few animals responding at low enzyme doses (Robinson *et al.*, 1996). The reliability of the ED50 calculation is improved by extending the dose–response profile to define the plateau regions of the dose–response curve. Ultimately, the suitability of the method is defined by its reliability. As detailed above, the use of the ED50 convention has produced relative potency determinations in good alignment with GPIT and human response data.

On the basis of these results, the MINT method is showing continual progress in its development as a valid alternative to the GPIT test for assessing the allergenicity of enzymes and potentially other proteins. Previous studies reported that the association between human and guinea pig allergic antibody responses to these enzyme allergens gives us some confidence that the GPIT could be useful in evaluating other classes of enzymes as well as nonenzyme proteins (Sarlo and Ritz, 1997). The correspondence between the MINT and the GPIT results supports extension of the MINT application as well. These applications could include other enzymes, food allergens, and, possibly, the preclinical allergenicity assessment of protein or peptide based therapeutics. Further work is underway to characterize airways symptomatology (Mendelson *et al.*, 1988) that may be produced by the sensitization procedure and thus useful in verifying the “allergic” character of the response measured. Studies have also been initiated to further expand the model to assess protease enhancement of the

potency of nonprotease enzymes as seen in the GPIT test (Robinson *et al.*, 1997; Sarlo *et al.*, 1997b). Ultimately, the MINT method may evolve as a standard approach for assessing enzyme and protein allergenicity.

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