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Research Section

Experience with a Mouse Intranasal Test for the Predictive Identification of Respiratory Sensitization Potential of Proteins

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Abstract—The predictive identification of respiratory allergenic potential is an important primary step in the safety evaluation of (novel) proteins, such as the enzymes used in a range of consumer laundry products. In the past this has been achieved by assessing the relative ability of proteins to give rise to the formation of anaphylactic antibody in the guinea pig. Recently, an alternative model has been proposed which assesses the formation of specific IgG1 antibody in a mouse intranasal test (MINT), the assumption being that specific IgG1 antibody is a surrogate for anaphylactic antibody in the mouse. This procedure has undergone successful initial intralaboratory and interlaboratory assessment. In the present work, the MINT has been evaluated in a more thorough intralaboratory study using eight enzymes plus ovalbumin. While the data generated with a reference enzyme protein, Alcalase, showed good reproducibility, results with the remaining eight proteins led to estimates of their relative antigenic or sensitization potential several of which were at variance from those derived from the guinea pig/human experience. In consequence, it is concluded that the MINT requires substantial further investigation before it can be adopted as a model for the assessment of the relative ability of proteins to behave as respiratory allergens. © 1999 Elsevier Science Ltd. All rights reserved

Keywords: respiratory; hypersensitivity; protein allergy; mouse intranasal test.

Abbreviations: ELISA = enzyme-linked immunosorbent assay; MINT = mouse intranasal test; OEGs = occupational exposure guidelines.

INTRODUCTION

The capacity of proteins, including detergent enzymes, to cause sensitization of the respiratory tract and thus to have the potential to induce asthma is well known (e.g. Douglas *et al.*, 1995; Flindt, 1969; Quirce *et al.*, 1998; Venables *et al.*, 1988). To control this phenomenon, it is necessary to identify to what extent particular proteins have such potential and then control human exposure appropriately. A major concern with the use of detergent enzymes is the risk of occupational allergy in the factory workforce. However, by strictly controlling the level of enzyme in the factory atmosphere using a combination of engineering controls and non-dusty enzyme encapsulates the incidence

rate of sensitization has been minimized (Juniper and Roberts, 1984). The proteolytic enzyme Alcalase has been used safely by the detergent industry for over 20 yr by compliance with empirically established occupational exposure guidelines (OEGs) based on experience and the deliberations of the American Conference of Governmental and Industrial Hygienists (ACGIH, 1990). Recommendations for target atmospheric levels for new enzymes are based on the allergenic potential of the new enzyme relative to that of the reference alkaline protease Alcalase as assessed in the guinea pig (Blaikie *et al.*, 1995; Ritz *et al.*, 1993), the assumption being that allergenicity is a direct function of antigenicity or sensitization potential. While the evidence confirming the predictive accuracy of the guinea pig for man is not extensive, there are data to suggest that indications of relative potency of

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protein respiratory allergens in that species are similar to those found in practice in man (Sarlo *et al.*, 1991, 1997).

Even though the guinea pig tests have proven to be useful, they are expensive and time consuming, and there is limited availability of specific immunological reagents, which causes difficulties, especially for mechanistic investigations. Consequently, the mouse intranasal test (MINT) was developed as an alternative method for the identification of the relative antigenic/sensitization potential of certain protein allergens, specifically detergent enzymes (Robinson *et al.*, 1996, 1998a). The MINT protocol is carried out in the B6D2F1 (BDF1) mouse strain using the intranasal route of exposure. As an endpoint, the development of antigen specific mouse IgG1 antibodies is measured, using an enzyme-linked immunosorbent assay (ELISA). IgG1 has been proposed by the originators of the MINT as a surrogate for the anaphylactic antibody IgE, the cytokine interleukin-4 being involved in the regulation of both isotypes (Purkerson and Isakson, 1992). The antigenic/sensitization potential of the test protein relative to that of the reference Alcalase is then assessed by comparison of the dose response profiles.

Our initial experience with the MINT in the context of an interlaboratory validation was positive (Blaikie *et al.*, 1997). Consequently, in the present work, the validity of the MINT as a predictor of the respiratory sensitization potential of proteins has been assessed with a more substantial dataset derived from existing studies in a guinea pig predictive model (Blaikie *et al.*, 1995), which itself has facilitated the definition of safe working conditions for the industries where the tested proteins have been employed (Juniper and Roberts, 1984; Unilever, unpublished data). The proteins tested included several types of proprietary detergent enzymes together with ovalbumin, a known human respiratory allergen (Bernstein *et al.*, 1987).

MATERIALS AND METHODS

Test materials

Seven proprietary enzymes (Novo Nordisk, Copenhagen, Denmark; Genencor, San Francisco, CA, USA) of both bacterial and fungal origin and different generic enzyme type (protease, cellulase, lipase and amylase) together with ovalbumin (A-5013, 99.8% protein; Sigma-Aldrich Company Ltd, Poole, Dorset, UK), all of which had previously been analysed in the guinea pig intradermal injection model (Blaikie *et al.*, 1995), were compared with the reference enzyme Alcalase (Novo Nordisk) in the MINT. The reference enzyme Alcalase was supplied by Procter & Gamble (Miami Valley Laboratories, Cincinnati, OH, USA), the originators of the MINT model as part of a collaborative

evaluation. A sample of Alcalase (Novo Nordisk) used in previous sensitization potency tests in the guinea pig was also compared with the reference Alcalase sample. The dosing solutions were prepared immediately prior to each dosing procedure at an appropriate range of concentrations on a protein weight basis in sterile isotonic saline containing 3 mg/ml of a detergent matrix. The detergent matrix consisted primarily of anionic and non-anionic surfactants, silicate builders and perborate bleach and was also kindly supplied by Procter & Gamble.

Animals

Groups of six female B6D2F1 (BDF1) mice weighing approximately 18–20 g at the start of the test were used. They were obtained from Harlan UK Ltd (Bicester, Oxon, UK) and were acclimatized for a minimum of 5 days prior to the start of the study. The mice were housed in groups of three in plastic boxes containing sawdust. The mice were fed pelleted diet (PCD, Special Diets Services, UK) and water *ad lib*. The suppliers of the diet provided information confirming that the PCD was ovalbumin free.

MINT protocol

The method followed that previously described (Robinson *et al.*, 1996). In brief, induction procedures were carried out on days 1, 3 and 10. The mice were anaesthetized and dosed with 10 μ l (5 μ l/nostril) of the appropriate dosing solution (test/reference material in detergent matrix). The dosing solutions were gently dispensed at the opening of each nostril as the animal inhaled. On day 15 the mice were exsanguinated and a serum sample was prepared from each blood sample for ELISA analysis of specific IgG1.

ELISA analysis

The serum samples prepared from the individual mouse blood samples were analysed for enzyme specific IgG1 antibodies using both antigen-capture (where antigen is bound directly to the microtitre plate) and antibody-capture (where the antigen in question is captured by a specific antibody bound to the microtitre plate) ELISA methods (Kawabata *et al.*, 1996; Robinson *et al.*, 1996, 1998a). Following optimization, the appropriate antigen or an antigen specific rabbit antisera (provided by the supplier of the specific enzyme or generated in-house) followed by the appropriate antigen was bound to the surface of plastic microtitre plates. Diluted serum samples (to provide a titration curve for each serum sample) were then added followed by a goat anti-mouse IgG1 alkaline phosphatase conjugated antibody (1/2000 dilution) (Southern Biotechnology Associates Inc, Birmingham, AL, USA) and finally *p*-nitrophenyl phosphatase substrate (1 mg/ml, w/v) (Kirkegaard & Perry Laboratories Inc, Gaithersburg, MD, USA). The

absorbance of the microtitre well contents were measured at 410/490 nm using an MR7000 Microplate Reader (Dynex Technologies Ltd, Billingshurst, West Sussex, UK).

Data analysis

A titration curve was prepared for each serum sample and the dilution corresponding to an optical density value of 0.5 was calculated. The results were expressed as reciprocal $\log_2$ titre values which were meaned for each dose group. Dose-response curves (reciprocal $\log_2$ titre versus enzyme dose) for each enzyme were plotted and the dose of enzyme required to give a 50% maximal response was determined (ED_{50}). In addition, the antigenic/sensitization potential of the test enzyme relative to that of the reference enzyme Alcalase was assessed by analysis of the dose-response curves, using both comparison of the ED_{50} values (Robinson *et al.*, 1996, 1998a) and by a statistically-based curve comparison routine. This method is derived from classical parallel line bioassays (Finney, 1980). In comparing two dose-response curves, the method establishes the regions over which the curves are rising almost linearly, are roughly parallel and for which there are the most data and compares the doses at various $\log_2$ titres over this range.

RESULTS

The proteolytic enzyme Alcalase was used as the reference enzyme for comparison in all cases and the dose responses to this enzyme showed inter-study consistency (Fig. 1). The results obtained using the antibody-capture ELISA method of analysis were quantitatively similar to those obtained using the antigen-capture ELISA method and biologically meaningful dose-response curves were generated in each case. In general, antibody-capture ELISA analysis provides more reliable results as more epitopes are exposed for binding. In the antigen-capture ELISA analysis, epitopes may be hidden as a result of antigen binding to the microtitre plate thus reducing the sensitivity of the assay. Therefore, unless otherwise specified, antibody-capture data have been cited. The results from the analysis of the reference enzyme Alcalase in 15 separate studies expressed as the mean (μg protein/dose/animal) $\pm$ standard deviation were 0.208 ± 0.079 using the curve comparison method and 0.232 ± 0.097 using the ED_{50} method. The results presented here were from studies for which the reference Alcalase curve was within the expected parameters.

The Alcalase sample used in previous sensitization potency tests in the guinea pig was analysed

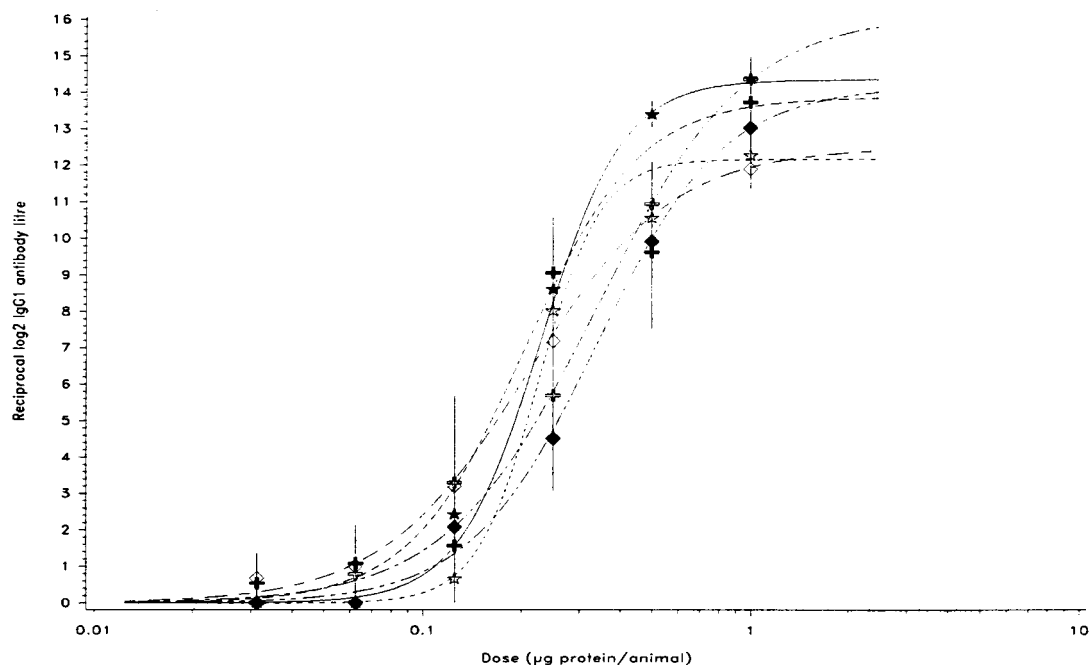


Fig. 1. Dose-response curves for the reference Alcalase sample from six representative studies demonstrating consistency. Using the curve comparison method the dose levels giving a reciprocal $\log_2$ titre of 6 are:

- ★ Study A 0.20 μg protein
- ☆ Study B 0.29 μg protein
- Study C 0.26 μg protein
- Study D 0.18 μg protein
- ◆ Study E 0.22 μg protein
- ◇ Study F 0.21 μg protein.

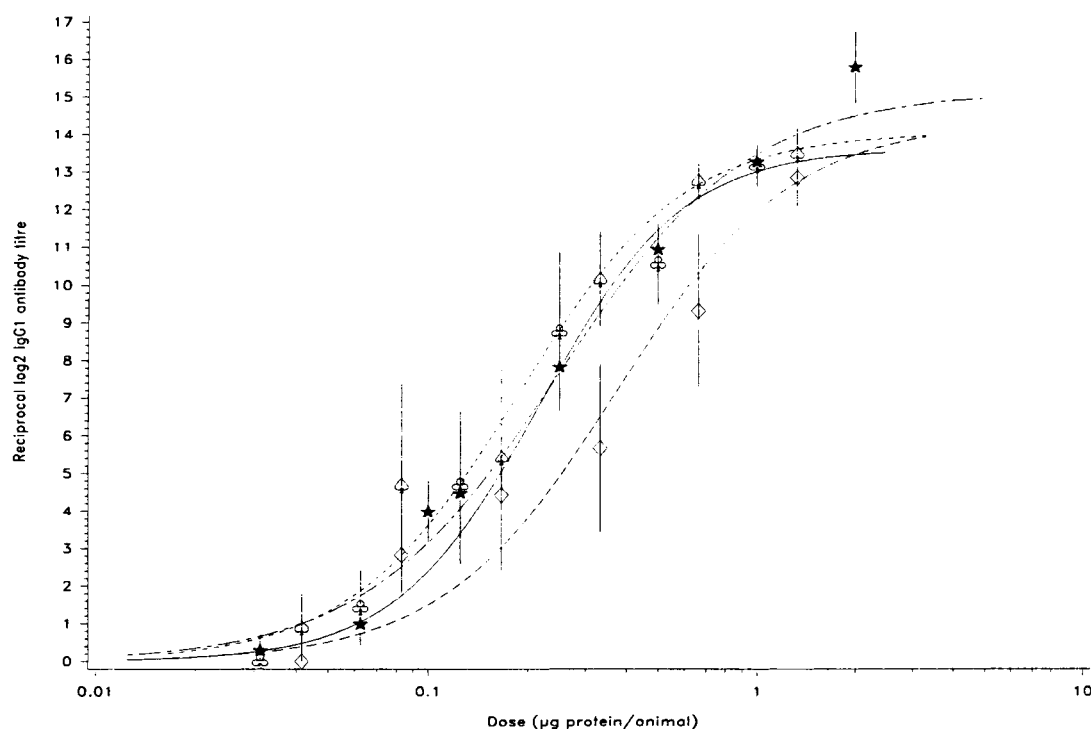


Fig. 2. Dose-response curves from three studies analysing the Alcalase sample used in previous antigenicity studies in the guinea pig demonstrating inter-study consistency and comparison to the reference Alcalase sample ★ (composite dose-response curve from 12 studies) used in all MINT studies. Relative antigenic potency (reference Alcalase:Alcalase) of 1.05 $\times$. Using the curve comparison method the dose levels giving a reciprocal log₂ titre of 6 are:

- ♣ Study X 0.19 μ g protein
- ♠ Study Y 0.12 μ g protein
- ◇ Study Z 0.22 μ g protein
- ★ Reference 0.19 μ g protein.

in three separate MINT studies (Fig. 2). The results expressed as the mean (μ g protein/dose/animal) $\pm$ standard deviation were 0.176 ± 0.053 using the curve comparison method and 0.212 ± 0.071 using the ED₅₀ method. The Alcalase sample used in previous sensitization potency tests in the guinea pig therefore gave similar results to the reference Alcalase sample used in the present work, with the relative antigenic/sensitization potential values being 1.18 $\times$ and 1.09 $\times$ using the curve comparison and ED₅₀ methods, respectively. These data indicate

that the different Alcalase samples produce similar responses in the MINT and justify the use of historical guinea pig data for comparison.

The results for the seven enzymes tested and ovalbumin are presented in Table 1. All eight of these proteins gave rise to the formation of specific IgG1 antibody and to biologically meaningful dose-response curves. The potency of these proteins in comparison to the reference Alcalase, has been expressed in terms of ED₅₀ values and also from the statistically-based curve comparison process. While these two methods of analysis give generally similar outcomes, it can be argued that the ED₅₀ approach is less sound scientifically than the curve comparison method. The ED₅₀ method is based on the examination of the upper asymptote (the upper limit to which the log₂ titre/dose-response curve) is converging, and the estimation of the dose which produces a log₂ titre of half the maximum (ED₅₀). Invariably the upper asymptote is an extrapolation and is therefore dependent on the fitting process and in consequence is not statistically robust. The curve comparison method which we have adopted uses an approach derived from classical parallel line bioassays. In contrast to the ED₅₀ approach, this

Table 1. Relative potency (reference:test) compared with the reference Alcalase

	Guinea pig	MINT
<i>Unilever Alcalase</i>	NA ¹	~1 $\times$
<i>Enzyme 1</i>	~3–10 $\times$	~0.3 $\times$
<i>Enzyme 2</i>	~3 $\times$	~6 $\times$
<i>Enzyme 3</i>	~3 $\times$	~2.5 $\times$
<i>Enzyme 4</i>	~1 $\times$	~1.2 $\times$
<i>Enzyme 5</i>	~1 $\times$	~0.1 $\times$
<i>Enzyme 6</i>	~1 $\times$	<0.1 $\times$
<i>Enzyme 7</i>	~1 $\times$	<0.1 $\times$
<i>Ovalbumin</i>	~3 $\times$	<0.1 $\times$

¹Not applicable as used as reference in the guinea pig model therefore assumed to be 1 $\times$.

method depends not on the unreliable extremes of the fitted curves but on the central regions of the curves for which there is the most information. In addition, where fitted dose-response curves do not plateau at the same level, the ED₅₀ comparison is inappropriate. Thus, the curve comparison method is considered to be statistically more sound. The ED₅₀ method of analysis was included to enable comparison of data with that generated by the originators of the MINT model (Robinson *et al.*, 1996, 1998a) as part of a collaborative evaluation (Blaikie *et al.*, 1997).

Nevertheless, whichever approach to data analysis is adopted, the results demonstrate that while there is reasonable correlation between MINT results and historical guinea pig data for some proteins (Enzymes 2, 3, 4), for others there is considerable divergence. Enzymes 5, 6 and 7 which were of similar antigenic/sensitization potential to the reference Alcalase in the guinea pig were substantially less antigenic in the BDF1 mouse. Another proprietary enzyme, Enzyme 1, together with ovalbumin, both of which were significantly and consistently more antigenic than Alcalase in the guinea pig, were also of considerably less antigenic/sensitization potential than Alcalase in the BDF1 mouse. Dose-response curves for selected enzymes are shown in Fig. 3(a-c). In each of two MINT studies, ovalbumin was substantially less potent (approx. 10-fold) than the reference Alcalase. This is illustrated in Fig. 4, which shows the data for ovalbumin. Judged against Alcalase, the potency differential between mouse and guinea pig assessments of the antigenicity of ovalbumin was approximately 30-fold.

DISCUSSION

The ability to predict the relative capacity of proteins to give rise to respiratory sensitization is a critical element in the safety evaluation of such materials. Guidance on suitable exposure limitations can only be made when such information is available. Consequently, considerable effort has been put into the development of reliable guinea pig models which are reasonably predictive of respiratory sensitization potential of protein allergens (Blaikie *et al.*, 1995; Ritz *et al.*, 1993; Sarlo and Karol, 1994; Sarlo *et al.*, 1991, 1997).

However, the guinea pig methodologies that have been utilized have certain drawbacks: they are relatively time-consuming and expensive, in some cases specialist equipment for the assessment of inhalation challenge reactions is needed, and the availability of reagents for investigative studies is limited. Consequently, effort has been put into the development of alternative approaches involving the mouse (Blaikie *et al.*, 1997; Hilton *et al.*, 1994, 1997; Kawabata *et al.*, 1996; Robinson *et al.*, 1996, 1998a). From this work, one protocol, the MINT,

has emerged as a possible candidate to replace the guinea pig methods used to assess the respiratory sensitization potential of detergent enzymes. It is this method that has been examined in detail in the present work.

The MINT protocol has been instituted successfully in our laboratory (Blaikie *et al.*, 1997). Indeed, the results using this method show a high level of reproducibility (Fig. 1) and this, together with the interlaboratory reproducibility observed with several enzymes (Blaikie *et al.*, 1997), gives a certain confidence in the data which are produced. However, the results presented in this paper indicate that while there is good correlation between the MINT data and the historical guinea pig data for certain proteins, for others there is considerable divergence. Although there is no firm evidence that the guinea pig model is superior to the mouse in predictive value for man, adherence to OEGs derived from guinea pig data has been successful in protecting the exposed workforce against asthma for many years (Flood *et al.*, 1985; Juniper and Roberts, 1984; Sarlo *et al.*, 1990). Moreover, the prospective evaluation of skin prick test data (prevalence rates of sensitization) for detergent workers exposed to different enzymes followed the predictions from the guinea pig data (Sarlo *et al.*, 1997). The evidence, therefore, is that the guinea pig has proven to be a reasonable model for the assessment of the antigenic/sensitization potential of enzymes and other proteins and should not be discarded lightly. Consequently, the suitability of the MINT as a direct replacement for the guinea pig methods is open to question.

The data from the evaluation of the MINT model which are inconsistent with the guinea pig results and/or human experience may reflect the problems associated with the use of an inbred or F1 hybrid mouse strain as well as inter-species differences. Such strains are known to show very different responses to individual antigens (Dandeu *et al.*, 1992; Holt *et al.*, 1981; Stewart and Holt, 1987). One of the events in the antigen recognition sequence involves the binding of peptides to Class II major histocompatibility (MHC) molecules (H-2 in the mouse) which are highly specific for each strain. Thus, it might be expected that different MHC molecules would bind peptides from the same antigen with different degrees of efficiency. Other biochemical differences in antigen handling (e.g. antigen processing by macrophages) together with other genetic factors such as the regulation of interleukin-4 (IL-4) production probably also account for differences in immune response between strains. The practical outcome of such considerations for the assessment of respiratory antigenic/sensitization potential of proteins in the mouse is reported elsewhere (Blaikie and Basketter, 1999; Blaikie *et al.*, 1998).

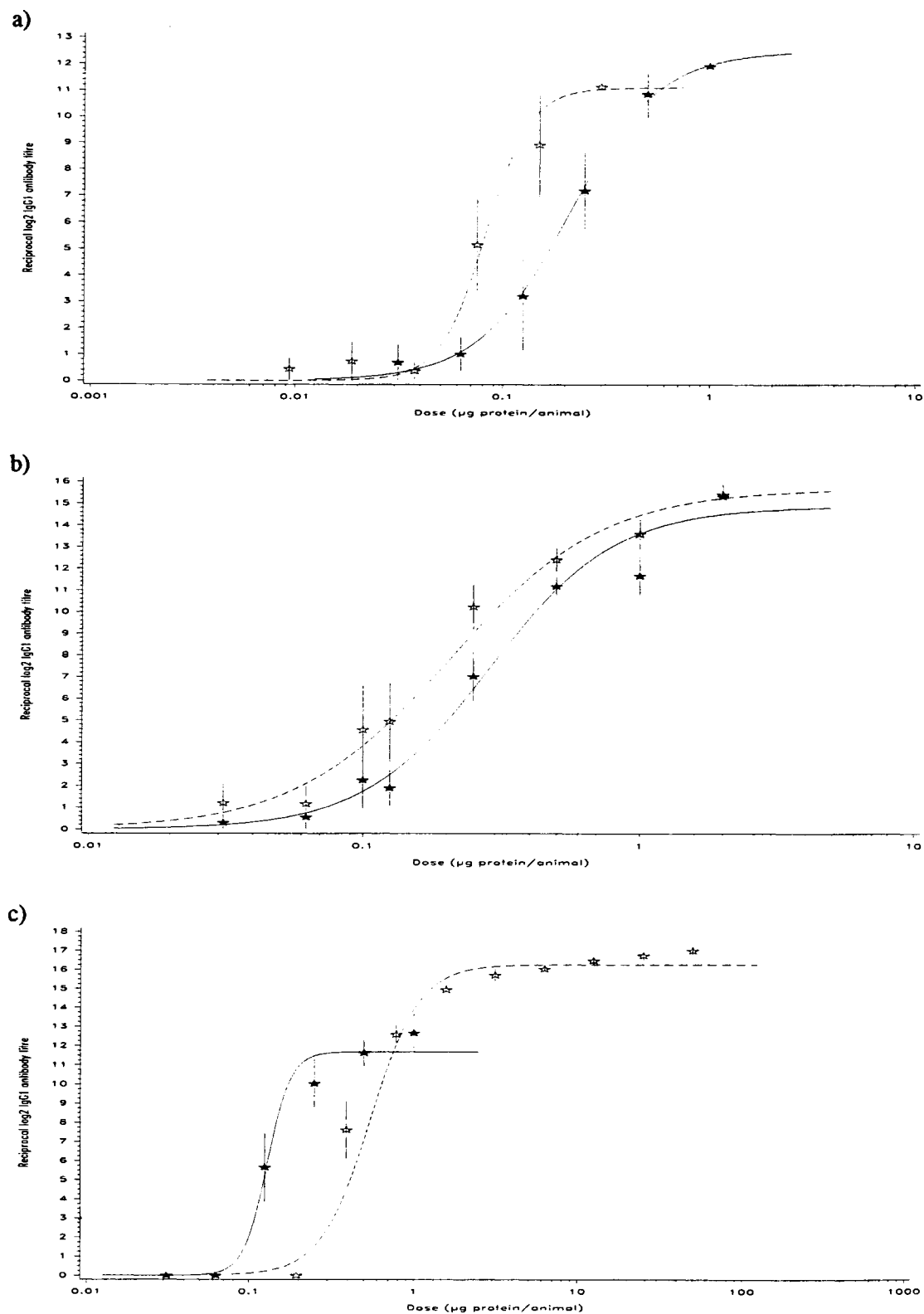


Fig. 3. (a) Comparison of dose-response curves for Enzyme 3 ☆ and reference Alcalase ★. Relative antigenic potency (reference Alcalase:Enzyme 3) of $2.5 \times$. (b) Comparison of dose-response curves for Enzyme 4 ☆ and reference Alcalase ★. Relative antigenic potency (reference Alcalase:Enzyme 4) of $1.2 \times$. (c) Comparison of dose-response curves for Enzyme 1 ☆ and reference Alcalase ★. Relative antigenic potency (reference Alcalase:Enzyme 1) of $0.33 \times$.

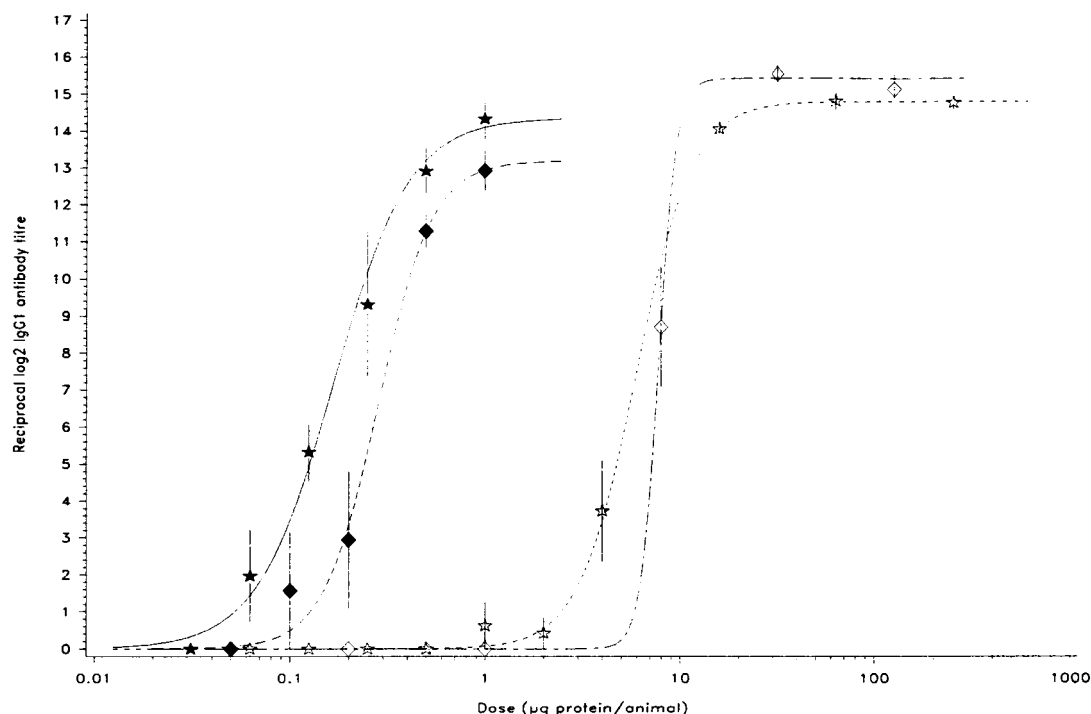


Fig. 4. Comparison of dose-response curves for ovalbumin (Study A ☆; Study B ◇) and reference Alcalase (Study A ★; Study B ◆) from two separate studies demonstrating inter-study consistency. Relative antigenic potency values (reference Alcalase:ovalbumin) of 0.028x and 0.034x, respectively.

A further concern about use of the MINT protocol for more general testing of the respiratory antigenic/sensitization potential of proteins is that the protocol as currently stated involves use of an adjuvant detergent matrix. The aim is to mimic the occupational exposure conditions found in detergent factories. However, it has already been shown that this adjuvant matrix has a differential (and unpredictable) effect on respiratory sensitizing potency (Robinson *et al.*, 1998b). This poses clear problems for general use of the present MINT protocol.

Currently in the MINT, specific IgG1 antibody is measured as a surrogate for IgE. Although there is some basis for this (Robinson *et al.*, 1996), not least that the production of both antibody isotypes is IL-4 dependent, there are data to show that IL-4 stimulation of IgG1 and IgE may be differentially regulated (Snapper *et al.*, 1988) and there are other complexities in the cytokine regulation of IgE (Purkerson and Isakson, 1992). Consistent with this is the observation that while ovalbumin, a significant respiratory allergen, evoked both IgG1 and IgE responses in Balb/c mice, bovine serum albumin, not regarded as a significant respiratory allergen, elicited equivalent IgG1 responses, but no detectable specific IgE (Hilton *et al.*, 1997). Before IgG1 can be used as a routine surrogate endpoint for IgE production in the mouse, the limitations of so doing need to be thoroughly understood in order to avoid erroneous interpretation of results.

In summary, based on evaluation of the data presented in this paper, the MINT requires substantial further investigation before it could be considered as a suitable model for the assessment of the relative antigenic/sensitization potential of protein allergens and therefore as a replacement for the guinea pig models currently used.

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