

## Immune Response to Bee Venom

## II. Quantitation of the Absolute Amounts of IgE and IgG Antibodies by Saturation Analysis

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**Abstract.** We have measured in allergic and non-allergic beekeepers IgE and IgG antibodies to the bee venom allergens, phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and hyaluronidase (HYAL), by the radioallergosorbent test (RAST) and a <sup>125</sup>I-radiolabelled antigen-binding assay. The absolute amount of IgG antibody in a reference serum was determined by saturation analysis using <sup>125</sup>I-radiolabelled PLA<sub>2</sub> and HYAL. Using monoclonal anti-IgE coated microtitre plates, the absolute amount of IgE antibody to the same antigen was also determined by saturation analysis. Regardless of the IgE response to the different allergens, IgG antibody concentrations to PLA<sub>2</sub> were invariably higher than those to HYAL. In addition, the ratio of IgG to IgE antibody was higher for PLA<sub>2</sub> (220:1) than for HYAL (10:1). Higher levels of IgG antibody to both allergens (especially HYAL) were found in those who had had prolonged exposure to bee stings. These data suggest that the level of IgG antibody produced is related to the dose administered, while the amount of IgE antibody may be regulated by other factors.

## Introduction

In patients who are allergic to bee stings, the highest levels of serum IgE antibodies are most frequently found to the major bee venom protein, phospholipase A<sub>2</sub> (PLA<sub>2</sub>), but are sometimes directed against other venom proteins, acid phosphatase (acid P), hyaluronidase (HYAL), or the peptide melittin (MEL) [1]. During venom immunotherapy, the production of IgG (blocking) antibodies is highly correlated with clinical protection from stings [2-6]. IgG antibodies in serum block the release of histamine from sensitised human leucocytes [7], and passively administered IgG antibodies to bee venom prevent or substantially reduce the reaction to subsequent venom challenge [8]. Nevertheless, in individual cases, production of IgG antibodies does not always mean clinical improvement and not all patients who are successfully treated produce high levels of IgG antibodies.

In a previous study [9], we compared the immune response to the major bee venom allergen, PLA<sub>2</sub>, with the response to the major grass pollen allergen, Rye I, and the major dust mite allergen, antigen P1. We found that there was invariably more IgG than IgE antibody and that the ratio of IgG/IgE antibody was

much higher for PLA<sub>2</sub> (mean 722:1) than for Rye 1 (mean 6.3:1) [10] or antigen-P1 (7.6:1) [11]. This study was undertaken to determine if these differences were a function of the route of administration or of the dose of antigen received by comparing the response to bee venom PLA<sub>2</sub> with that to HYAL (bee venom contains approximately 10 times more PLA<sub>2</sub> than HYAL). The problem of accurately measuring IgE antibody in the presence of an excess of IgG antibody to the same antigen was overcome by using mouse monoclonal anti-IgE-coated microtitre plates to separate IgE from IgG prior to saturation with <sup>125</sup>I-radio-labelled antigen.

## Materials and Methods

## Patients Studied

The patients studied were either non-allergic beekeepers (28) or had a history of generalised (28) or local (29) allergic reactions to bee stings but had not been given bee venom immunotherapy. A generalised reaction to not more than 5 simultaneous stings was recorded when the patient had exhibited one or more of the following, within the past 3 years: (i) generalised skin rash or swelling; (ii) wheezing or difficulty in breathing; (iii) swelling of the tongue or throat; (iv) vomiting, diarrhoea, or abdominal pain, and (v) collapse. A local allergic reaction to a single sting was recorded when

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there was a swelling of greater than 2 inches in diameter continuous with the site of the sting. The non-allergic beekeepers had had no allergic reaction to bee stings within the preceding 3 years.

#### Bee Venom Antigens

Highly purified bee venom hyaluronidase was prepared as described previously [12, 13]. Phospholipase A<sub>2</sub> was prepared by Dr. R.A. Shipolini [14], Imperial College London, and was a kind gift from Prof. C.A. Vernon, University College London. Both proteins were judged to be pure by polyacrylamide gel electrophoresis (PAGE) [15]. The preparation of HYAL contained <0.1% PLA<sub>2</sub>, and the PLA<sub>2</sub> preparation showed no detectable HYAL activity.

#### Radiolabelling Procedure

PLA<sub>2</sub> and HYAL were radiolabelled with <sup>125</sup>NaI (Radiochemical Centre, Amersham) by a modified chloramine T method [16, 17]. Briefly, 50 µg of protein were dissolved in 50 µl of 0.25 M, pH 7.4 sodium phosphate buffer and mixed with 0.5 MCi of <sup>125</sup>NaI in a small glass tube over crushed ice. Chloramine T (50 µl of 2 mg/ml solution in phosphate buffer) was added and the tube gently rotated for 1 min, followed by sodium metabisulphite (50 µl of 2 mg/ml solution in phosphate buffer) for 2 min. Finally, 50 µl of sodium iodide (1% w/v in phosphate buffer) and 250 µl of 0.2% w/v bovine serum albumin (BSA) in phosphate buffer, were added. Free <sup>125</sup>I was separated from <sup>125</sup>I-radiolabelled-protein by passage over a column (1 × 10 cm) containing Sephadex G-50, medium grade (Pharmacia GB, Ltd) which was equilibrated in phosphate buffer containing 0.2% BSA - to minimise adsorption of the radiolabelled protein to the column. The specific activity of the radiolabelled PLA<sub>2</sub> was 6.02 µCi/µg and of the HYAL, 2.76 µCi/µg. The radiolabelled proteins were aliquoted in 250-µl lots and stored at -20°C for up to 2 months.

#### Antigen-Binding Assay

<sup>125</sup>I-radiolabelled antigen-binding assays have been extensively used to measure class-specific human antibodies [5, 18, 19]. Maximum sensitivity is achieved by the use of small quantities of <sup>125</sup>I-radiolabelled antigen. Similar assays have already been used to measure IgG antibodies to Rye I [20] from Rye grass pollen, and P1 from *Dermatophagoides pteronyssinus* [21]. The assay described here utilises protein A-coated Sepharose 4B [22, 23] (Pharmacia GB, Ltd) to separate the <sup>125</sup>I-radiolabelled antigen-antibody complex from free <sup>125</sup>I-radiolabelled antigen. Using small quantities of antigen (1-3 ng), most sera were assayed at 1/200 or greater dilution, thereby minimising the binding of IgE to the protein A-Sepharose 4B. When we incubated 10 µl of <sup>125</sup>I-IgE from the Pharmacia IgE test kit with 0.5 mg of protein A-Sepharose 4B overnight, only 2.9% of added counts bound to the protein A-Sepharose 4B. Further, two sera which had a high level of IgE antibody to HYAL but very little IgG antibody to HYAL, gave a low level of binding to protein A-Sepharose 4B, suggesting that IgE antibody does not affect the IgG assay.

The immunoreactivity of both antigens was high and excess antibody bound 93% of added PLA<sub>2</sub> or 90% of added HYAL. The assay was performed as follows: 100 µl of test serum at a 1/200 dilution in 0.05 M phosphate-buffered saline/BSA (assay diluent) (pH 7.4, 0.9% NaCl, 0.2% BSA (w/v) and 0.5% Tween 20 (v/v) (Sigma Ltd) were incubated with 100 µl of <sup>125</sup>I-radiolabelled antigen (1-3 ng) in 100 µl of the same diluent for 4 h at room temperature (25°C) in LP3 tubes (Luckham Ltd). Protein A-Sepharose 4B

(0.5 ml, 1 mg/ml) was added, the tubes stoppered and mixed end-over overnight at room temperature. Unbound <sup>125</sup>I-radiolabelled antigen was removed by 5 washes of 2 ml phosphate-buffered saline (0.05 M, pH 7.4, 0.9% w/v NaCl) containing 0.05% Tween 20. The tubes were centrifuged for 1 min at 2,000 rpm in an MSE 6 L centrifuge in between each wash and counted for 1 min in an LKB multichannel gamma counter. Results were expressed as units by reference to a reference serum taken from a hyperimmune beekeeper (IgG reference serum) which was found to contain 24 µg/ml of IgG anti-PLA<sub>2</sub> and 7.8 µg/ml IgG anti-HYAL, by saturation analysis, and which was assigned 10,000 arbitrary units of IgG anti-PLA<sub>2</sub>/ml. Variation within assays was <5.0% and between assays <10%, within the straight portion of the standard curve. The units of IgG anti-HYAL were adjusted to correspond to the same quantity of IgG antibody as the units of IgG anti-PLA<sub>2</sub>. Sera with very low levels of IgG antibody were re-assayed at 1/50 or 1/100 dilution, and those with a very high concentration of antibody at 1/500 or 1/1,000. Binding below 1.5 times that of a 1/50 dilution of normal human serum was not considered significant and was scored ND (not detectable).

#### Quantitation of IgG Antibody by Saturation Analysis

The procedure used to determine the exact quantity of IgG anti-PLA<sub>2</sub> and HYAL in the IgG reference serum was essentially the same as that described previously [24, 25]. The quantity of <sup>125</sup>I-radiolabelled antigen bound by the reference serum at different dilutions was measured in antigen excess. The immunoreactivity of the antigen is high, and assuming that in gross antigen excess all the antibody would exist in immune complexes in the form of AB-Ag<sub>2</sub>, the quantity of specific IgG antibody can be calculated from the following equation [25]:

$$\text{Ng specific IgG per assay tube} = \frac{\text{net cpm bound in antigen excess plateau}}{\text{specific radioactivity of immune reactive antigen}} \times \frac{\text{molecular weight of IgG}}{2 \times \text{molecular weight of antigen}}$$

#### Measurement of IgE Antibodies

IgE antibodies to PLA<sub>2</sub> and HYAL were measured by the radio-allergosorbent test (RAST) as described previously [1]. To ensure that the assay was antigen-specific, the quantity of HYAL used to coat the cyanogen bromide-activated discs was kept low (10 µg per 500 discs). The specificity of the PLA<sub>2</sub> RAST was not found to be a problem but, because of difficulties through competition with IgG anti-PLA<sub>2</sub> [26], a larger amount (100 µg) was used. To calibrate the PLA<sub>2</sub> and HYAL RAST, the reference serum was calibrated by saturation analysis as for IgG antibody. Because IgG antibody that was present in the serum at a higher level, IgE was first bound to 'removawell' microtitre plates (Dynatech M129A, UK) coated with mouse-monoclonal anti-IgE (1 µg/ml in pH 9.6 carbonate, bicarbonate buffer 0.1 M, overnight at 4°C), kind gift from Prof. A. Saxon, UCLA, USA. Briefly, 100 µl of reference serum diluted 1/10-1/200 were incubated with the anti-IgE-coated microtitre plate in horse serum (Sera lab, UK) overnight at 4°C. Control wells were incubated either with <sup>125</sup>I-IgE (Pharmacia RIA) to determine what proportion of IgE had bound or with hyperimmune beekeeper serum to assess non-specific binding of IgG antibody. Unbound material was washed off with PBS/0.05% Tween 20. <sup>125</sup>I-radiolabelled PLA<sub>2</sub> or HYAL was then added, diluted in horse serum,

**Table I.** Effect of the quantity of  $^{125}\text{I}$ -radiolabelled antigen on the sensitivity of the antigen-binding assay. The optimal discrimination between the IgG reference serum at 1/2,000 and normal human serum at 1/100 was at 20,000 cpm (1.31 ng) for PLA and at 10,000 cpm (1.81 ng) for HYAL. The % values refer to the % of added cpm

| ng PLA <sub>2</sub><br>per tube | % cpm<br>normal<br>human serum<br>1/100 | % cpm<br>IgG antibody<br>serum<br>1/2,000 | Ratio of IgG<br>reference<br>serum/normal<br>human serum |
|---------------------------------|-----------------------------------------|-------------------------------------------|----------------------------------------------------------|
| 0.83                            | 1.33                                    | 28.7                                      | 21.6:1                                                   |
| 1.31                            | 0.93                                    | 21.1                                      | 27.7:1                                                   |
| 3.48                            | 0.51                                    | 9.60                                      | 18.8:1                                                   |
| 4.53                            | 0.62                                    | 7.71                                      | 12.4:1                                                   |
| 6.1                             | 0.47                                    | 6.21                                      | 13.2:1                                                   |
| 7.19                            | 0.35                                    | 5.04                                      | 14.1:1                                                   |
| <b>ng HYAL<br/>per tube</b>     |                                         |                                           |                                                          |
| 1.81                            | 1.87                                    | 13.39                                     | 7.16:1                                                   |
| 3.93                            | 1.22                                    | 6.98                                      | 5.72:1                                                   |
| 6.97                            | 0.96                                    | 4.43                                      | 4.61:1                                                   |
| 9.77                            | 0.73                                    | 4.13                                      | 5.66:1                                                   |
| 14.31                           | 0.77                                    | 3.60                                      | 4.67:1                                                   |
| 17.70                           | 0.89                                    | 3.10                                      | 3.48:1                                                   |

and following a further overnight incubation, unbound radiolabel was washed off and the bound antigen measured in a gamma counter (LKB, multichannel). No binding of IgG antibody was observed even though it was present in a 100-fold excess. The efficiency with which the monoclonal anti-IgE-coated wells bound IgE was determined by addition of  $^{125}\text{I}$  IgE (Phadebas RIA kit - Pharmacia Plc) and was >95% for the range of serum dilutions tested.

#### Statistical Analyses

The level of specific antibody in the different groups was compared by the paired or unpaired Student's *t* test. The levels of IgE and IgG antibody were compared by linear regression.

## Results

#### Determination of Optimum Antigen Concentration

The effect of antigen concentration was tested over a range of dilutions of antibody (IgG reference serum). The results, expressed as percentage counts per minute (cpm) bound, are given in table I. The less radiolabelled antigen added the greater the sensitivity. The highest signal (antibody) to noise (background), was achieved using 1.31 ng PLA<sub>2</sub> and 1.81 ng HYAL. Intra-assay variation did not alter significantly over the range of antigen concentrations used.

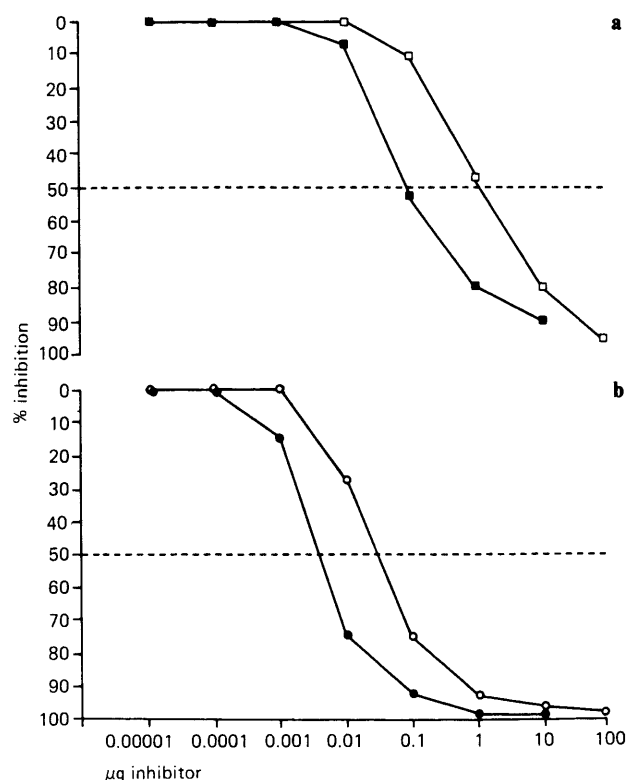


**Fig. 1.** **a** Inhibition of  $^{125}\text{I}$ -HYAL binding to IgG antibody by cold HYAL (■) or bee venom (□). Cold PLA<sub>2</sub> was completely unable to inhibit binding. **b** Inhibition of  $^{125}\text{I}$ -PLA<sub>2</sub> binding to IgG antibody by cold PLA<sub>2</sub> (●) or bee venom (○). Cold HYAL was completely unable to inhibit binding.

#### Specificity of the Assay

To ensure that the assays for IgG antibodies to PLA<sub>2</sub> and HYAL were specific for antigen, cold antigen was used to inhibit binding of the  $^{125}\text{I}$ -radiolabelled antigens to IgG antibody. The IgG reference serum was diluted so that approximately half of the added counts were bound (1/700 for  $^{125}\text{I}$ -PLA<sub>2</sub>, 1/500 for  $^{125}\text{I}$ -HYAL). Ten-fold dilutions of cold antigen were mixed with radiolabelled antigen, and appropriately diluted IgG reference serum added. After 4 h, 0.5 ml of protein A-Sepharose 4B (1 mg/ml) was added and the assay continued as described earlier. The percentage inhibition of binding was plotted against micrograms of added inhibitor (PLA<sub>2</sub>, HYAL, or HBV). Cold PLA<sub>2</sub> was unable to inhibit binding of  $^{125}\text{I}$ -radiolabelled HYAL (fig. 1a), and cold HYAL did not inhibit binding of  $^{125}\text{I}$ -radiolabelled PLA<sub>2</sub> (fig. 1b). Both assays were, therefore, judged to be highly specific.

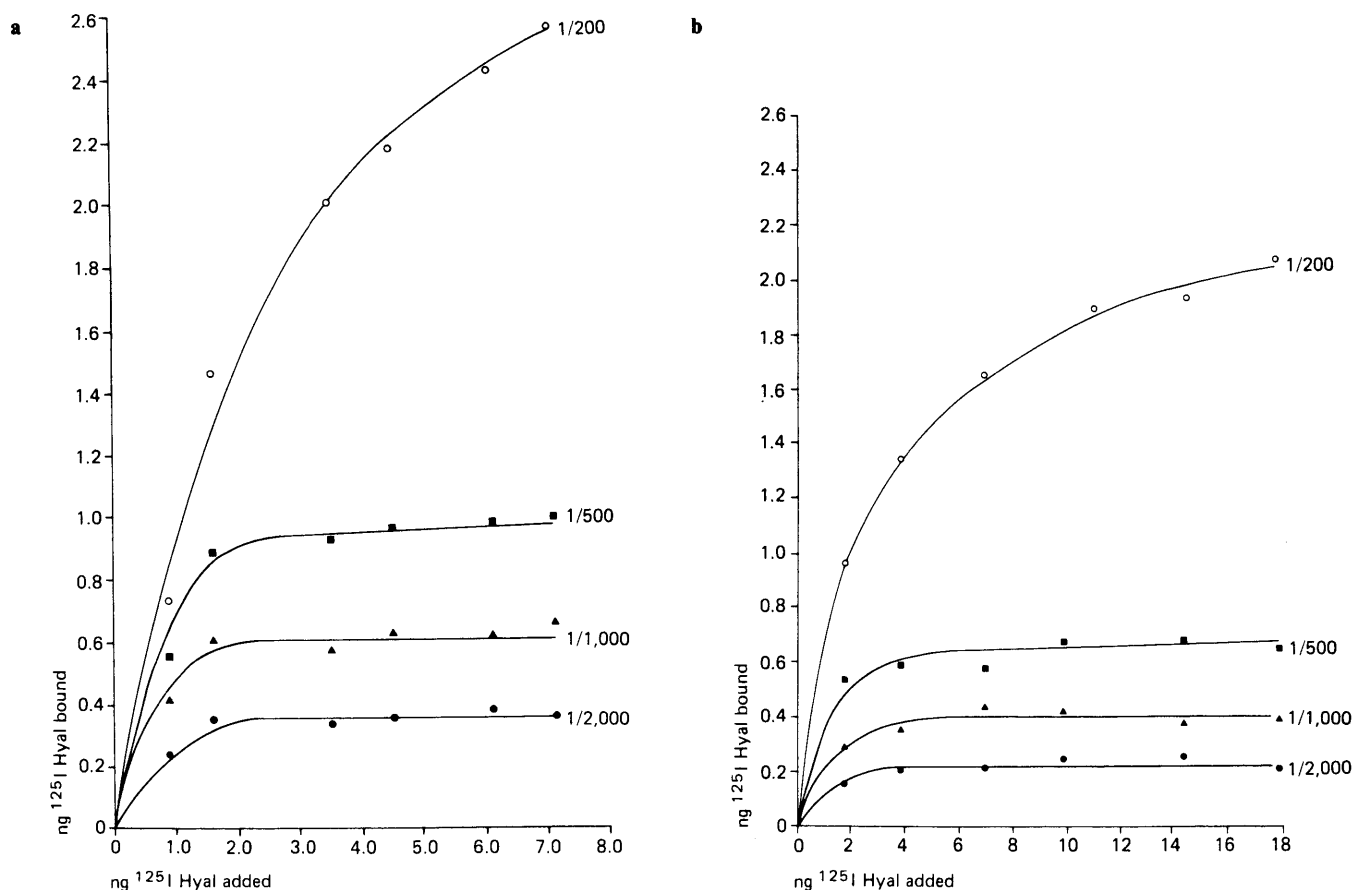


Fig. 2. Estimation of the quantity of IgG antibody to PLA<sub>2</sub> (a) and HYAL (b) by saturation analysis with (●) 1/2,000 (▲) 1/1,000 (■) 1/500 dilution of IgG reference serum. At (○) 1/200 no plateau was reached in antigen binding.

#### Quantitation of IgG Antibody by Saturation Analysis

The absolute quantity of IgG antibody to PLA<sub>2</sub> and HYAL was estimated by saturation analysis at 1/2,000, 1/1,000 and 1/500 dilution (fig. 2a,b). Above 1/500, saturation was not reached with 7.19 ng of PLA<sub>2</sub> or 17.7 ng of HYAL. Using the equation detailed in Materials and Methods, the quantity of IgG antibody was estimated at each antibody dilution (1/2,000, 1/1,000, 1/500) and the mean value calculated. The IgG reference serum was found to contain 24 µg/ml IgG anti-PLA<sub>2</sub> and 7.8 µg/ml anti-HYAL.

#### Quantitation of IgE Antibody by Saturation Analysis

The absolute amount of IgE antibody in the reference serum was estimated as above except that the IgE antibody was bound to monoclonal anti-IgE-coated

microtiter plates. This meant that IgE antibody was measured without IgG antibody being present. The results, figure 3a,b, show that it was possible to saturate both PLA<sub>2</sub> and HYAL. Saturation with HYAL resulted in a fall in binding when the largest amount of HYAL was used due possibly to a disproportionate increase in non-specific binding of radiolabelled antigen (which was generally very low -0.6-0.18% cpm). Extrapolation from the plateau of the curves showed the IgE reference serum to contain 193 ng/ml IgE anti-PLA<sub>2</sub> and 27 ng/ml IgE anti-HYAL. Control wells containing hyper-immune beekeeper serum, which had a high level of IgG but no IgE antibody, were negative.

#### IgG Antibodies to PLA<sub>2</sub> and HYAL

IgG antibodies to PLA<sub>2</sub> and HYAL were measured in the sera of allergic and non-allergic beekeepers.

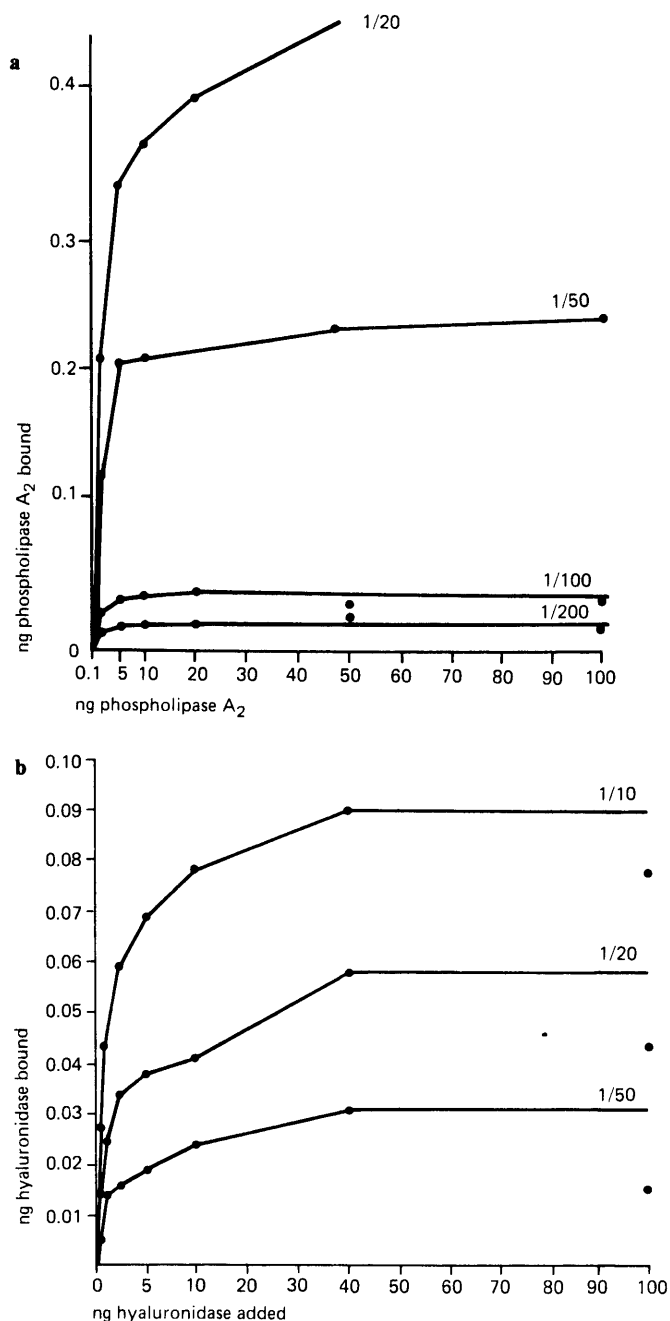


**Fig. 3.** Estimation of IgE antibody to  $\text{PLA}_2$  (a) and HYAL (b) by saturation with  $^{125}\text{I}$ -radiolabelled antigen. Very high concentrations of HYAL produced an apparent reduction in the plateau caused by higher background binding.

The majority of those studied possessed detectable levels of IgG antibodies to both antigens (fig. 4). Higher levels of IgG antibody to  $\text{PLA}_2$  than to HYAL were found in all subjects studied ( $p < 0.001$  Matched pairs t test). The ratio of  $\text{PLA}_2$ :HYAL IgG antibody was

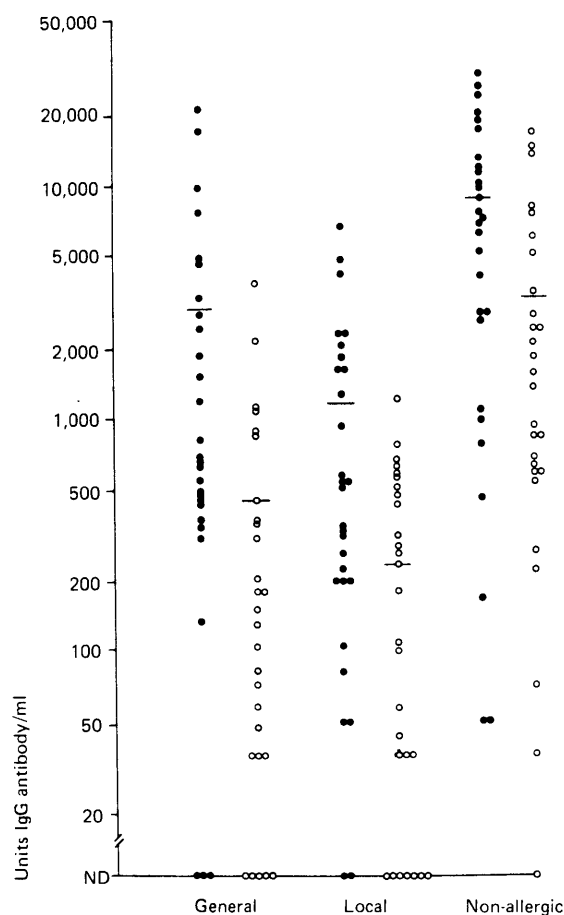


**Fig. 4.** IgG antibody to  $\text{PLA}_2$  (●) and HYAL (○) in sera taken from bee sting allergic patients (general and local reactors) and non-allergic beekeepers.

15:1 in the venom-allergic patients and 5.8:1 in the non-allergic beekeepers, although there was more IgG antibody to both antigens in the non-allergic compared with the allergic beekeepers (t test  $p < 0.01$ ). There was a correlation between the level of IgG antibody to  $\text{PLA}_2$  and HYAL ( $r = 0.78$   $p < 0.01$ ).

#### *Relationship between IgG and IgE Antibodies*

There was no correlation between the amount of IgG and IgE antibody to  $\text{PLA}_2$  ( $r = -0.156$ ) or HYAL ( $r = -0.092$ ). However, relatively more IgG than IgE antibody was produced against  $\text{PLA}_2$  (mean ratio IgG IgE antibody 220:1) compared with HYAL (mean ratio IgG:IgE antibody 10:1) (fig. 5). These differences reflect the 5- to 10-fold larger quantity of  $\text{PLA}_2$  found in bee venom.

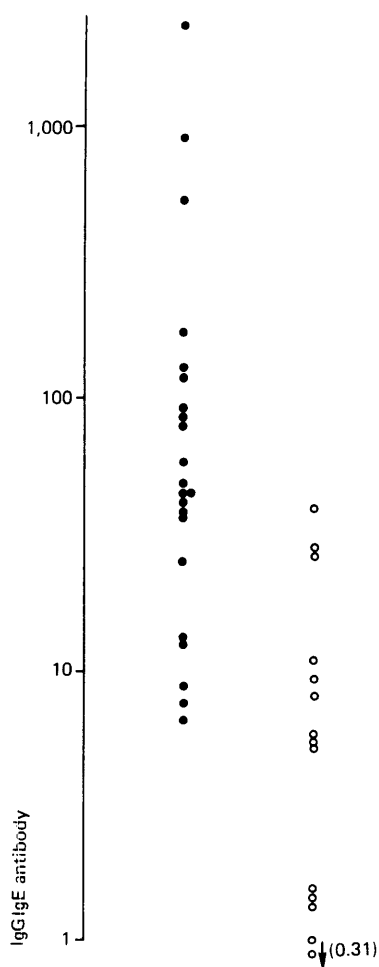


Fig. 5. Comparison of the relative quantities of IgG and IgE antibody to PLA<sub>2</sub> (●) and HYAL (○) in patients who had had generalised allergic reactions to bee stings.

## Discussion

The efficacy of vaccines used for therapy is closely correlated with their ability to stimulate a strong IgG antibody response [2–6]. In keeping with the view that vaccine efficacy depends upon immunogenicity [27], bee venom is much more effective than whole bee body extract [28]. Nevertheless, there is not a simple relationship between the amount of IgG antibody produced and clinical protection. Some patients who are successfully treated (and shown to be protected) do not show significant increases in IgG antibody while others whose IgG antibodies rise may still be clinically sensitive [32]. In previous studies [1, 33] we have shown that patients may be more sensitive to

some bee venom components than to others. This study was undertaken to determine the magnitude of the IgG level compared with the IgE response to these antigens.

Precise quantitation of IgE and IgG antibodies to PLA<sub>2</sub> and HYAL presented a number of problems. First, the proportion of total serum IgE that is specific for the venom antigens is generally smaller than for inhalant antigens. RAST absorption [34] or elution [35] can be used where a substantial proportion of the IgE is specific for a single allergen (25–30% or more) but are not appropriate here as only a small percentage of IgE is venom-specific. Saturation analysis has been used extensively to quantitate IgG antibodies [24, 25] but rarely to measure IgE antibodies [31]. As we have shown in this study, the coexistence of large amounts of IgG antibodies in the assay mixture can interfere with the binding of IgE antibodies. This can be overcome by prior separation of IgE and IgG and this was achieved with monoclonal anti-IgE-coated microtitre plates.

The majority of bee venom-allergic patients and non-allergic beekeepers studied possessed IgG antibodies to both PLA<sub>2</sub> and HYAL. As we [32] and others [29] have shown previously, non-allergic beekeepers produce more venom-specific IgG antibodies than do bee venom-allergic patients – presumably a reflection of greater exposure to stings. In all subjects studied, there was more IgG antibody directed against PLA<sub>2</sub> than against HYAL although the differential (IgG PLA<sub>2</sub>:HYAL) was greater in the bee venom-allergic patients (15:1) compared with the non-allergic beekeepers (5.8:1). Golden et al. [30] reported that up to a maximum level, the amount of IgG antibody produced during venom immunotherapy was a function of the cumulative dose given. When a vaccine is given which contains a mixture of antigens, our results suggest that the production of antibody to the individual components may also reflect the dose given. Thus, patients who are most sensitive to minor allergens, such as hyaluronidase, may require more injections or a higher dose regimen to achieve the same level of immunity.

In a previous study [9] a comparison of the relative amount of IgG and IgE antibody to PLA<sub>2</sub> showed that there was, on average, 722 times more IgG than IgE antibody, while inhalant antigens such as *D. pteronyssinus* and rye grass [10, 11] produced relatively less IgG antibodies (IgG:IgE, 7.6:1 and 6.3:1, respectively). Using the saturation analysis-calibrated

RAST described in this study we again found a high ratio of IgG to IgE for PLA<sub>2</sub> (220:1). The slightly lower ratio obtained in the present study suggests that there may have been competition between IgG and IgE antibody for <sup>125</sup>I-radiolabelled antigen in the antigen-binding assay we used previously [9]. The 10:1 ratio of IgG:IgE antibody to HYAL which we obtained was, however, much closer to that found with inhalant antigens. Although this may depend to some extent on the dose of antigen administered, grass pollen and dust mite allergic patients differed from bee venom allergic patients by showing a close correlation between IgG and IgE antibody levels which bee venom allergic patients did not. It is possible that those who develop an allergy to inhalant antigens may develop a greater hypersensitivity (and a greater IgG response) with increasing exposures – possibly due to enhanced mucosal permeability. Whether or not this is so, repeated exposure to bee venom shows an inverse relationship to clinical sensitivity, since further exposure is avoidable and allergic patients try to avoid being stung.

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