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## COMPARISON OF BINDING OF IgE AND IgG ANTIBODIES TO HONEYBEE VENOM PHOSPHOLIPASE-A<sup>1</sup>

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We compared the quantities and avidities of IgE and IgG antibodies from three phospholipase-A (PLA) sensitive patients during honeybee venom immunotherapy. IgG antibody was purified by affinity chromatography through a Sepharose anti-IgE column followed by ion-exchange chromatography on DEAE Sephadex. IgE antibody was obtained by elution from the Sepharose anti-IgE column with 4 M guanidinium HCl and by passage of the eluate over Sepharose anti-IgG. Absolute amounts of IgE and IgG antibody to PLA were determined by immunoabsorption and radioimmunoprecipitation, respectively. IgE antibody to PLA accounted for 25.1% to 28.7% ( $\bar{X}$  = 27.0%) of the total IgE protein; IgG antibody accounted for 0.073% to 0.210% ( $\bar{X}$  = 0.130%) of the total IgG protein. The relative avidities of IgE and IgG antibodies were assessed by the ability of equimolar amounts to bind radioiodinated PLA. In two patients, avidities of IgE and IgG antibody for PLA were comparable, whereas in the third, IgG antibody bound PLA more avidly than IgE antibody. The valence of IgE antibody was greater than 1.9, assuming that PLA behaved as a univalent ligand in extreme antigen excess. IgG antibody was able to inhibit binding of IgE antibody in the PLA radioallergosorbent test (RAST) from 10% to 40% at a molar excess of 10- to 1000-fold. The results indicate that: 1) IgE antibody to PLA accounts for a high percentage of total IgE protein; 2) the avidities of IgE and IgG antibodies are similar (two of three patients); 3) the valence of IgE antibody approaches two; and (4) IgG antibody can interfere with the measurement of IgE antibody in the PLA RAST.

In patients with allergic rhinitis, IgE antibodies often account for high percentages of total IgE protein (1-3). In contrast, IgG antibodies are present in small amounts unless the patient has received parenteral injections of allergens. IgG antibodies have

been considered important in allergic subjects because of their ability to block allergic reactions *in vitro* (4); moreover, with the advent of *in vitro* tests for serum IgE antibodies such as the radioallergosorbent test (RAST)<sup>3</sup> (5) one must be aware that IgG antibodies might possibly interfere in the procedure. The levels of IgG and IgE antibodies to phospholipase-A (PLA) appear to have clinical importance in most honeybee venom (HBV)-sensitive people (6-8). Most sensitive subjects have high levels of IgE antibodies to PLA and after HBV immunotherapy the levels of IgG antibody rise in association with clinical protection (9, 10).

In this study we investigated IgE and IgG antibodies to PLA formed in response to HBV immunotherapy in three allergic people. We describe a technique for purification and quantitation of IgG and IgE antibodies to PLA. The quantities and relative avidities of the two antibodies for PLA were compared, and the valence of IgE antibody to PLA was estimated. Finally, the ability of IgG antibody to PLA to interfere with the binding of IgE antibody to PLA in the RAST has been documented.

### MATERIALS AND METHODS

**Sera.** Sera were obtained by plasmapheresis (approximately 1 month after the previous injection) from three HBV-sensitive patients undergoing honeybee venom immunotherapy. Patient 1 received a total cumulative dose of 744  $\mu$ g of HBV during two 3-day courses of rush immunotherapy. Patient 2 received 1090  $\mu$ g of HBV during a single 2-day rush-injection program, and patient 3 received a cumulative dose of 988  $\mu$ g of HBV during two 2-day rush courses of immunotherapy. All patients had sustained one or more life-threatening anaphylactic episodes after a honeybee sting. Two patients had previously received honeybee whole-body extract immunotherapy with no apparent benefit, because anaphylactic reactions followed subsequent honeybee stings. Sera were also obtained from six healthy non-hyposensitized patients to serve as a control serum pool. Fibrinogen was removed by dialysis with 0.01 M CaCl<sub>2</sub> in 0.15 M NaCl followed by dialysis with 0.15 M NaCl alone.

**PLA.** PLA was prepared as described previously (11). Briefly, HBV grade I (Sigma Chemical Co., St. Louis, Mo.) was fractionated by gel filtration through a calibrated 2.5 x 200 cm Sephadex G-75 column (Pharmacia Laboratories, Piscataway, N.J.) equilibrated and eluted with 0.1 M ammonium formate (pH 4.5). PLA fractions corresponding to the 277-nm absorbance peak at approximately 14,000 to 16,000 daltons were pooled and further purified by ion-exchange chromatography with a 2.5 x 22 cm CM-Sephadex column (Pharmacia Laboratories).

<sup>3</sup> Abbreviations used in this paper: RAST, radioallergosorbent test; PLA, phospholipase-A; HBV, honeybee venom; phosphate buffer, K<sub>2</sub>HPO<sub>4</sub>-KH<sub>2</sub>PO<sub>4</sub>; DARIA, double-antibody radioimmunoassay; RIP, radioimmunoprecipitation; S2B, Sepharose 2B; HSA, human serum albumin; Cyto c, cytochrome c; CM, carboxymethyl.

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eluted with a linear gradient of 0.1 to 1.5 M ammonium formate (pH 4.75).

**Antiserum to human IgE.** Antiserum to human IgE was prepared as previously described (12). Briefly, myeloma IgE (PS) was digested with papain and the fractions containing Fc fragments were isolated by Sephadex G-200 chromatography. Burro anti-IgE (Fc) was raised and absorbed with IgE-deficient human serum covalently linked to Sepharose 2B (S2B). The burro anti-IgE (Fc) was further purified by a 28% Na<sub>2</sub>SO<sub>4</sub> (w/v) precipitation. The resulting anti-IgE (Fc) preparation yielded a single band when analyzed with myeloma IgE (PS) and was coupled to S2B as described previously (13).

**Antiserum to human IgG.** Human IgG (Cohn fraction II, American Red Cross) was fractionated by ion-exchange chromatography through a 2.5 x 22 cm column of DEAE-cellulose (DE-32, Whatman Inc., Afton, N.J.) and a protein peak of IgG was obtained in fall-through fractions with 0.005 M K<sub>2</sub>HPO<sub>4</sub>-KH<sub>2</sub>PO<sub>4</sub> (phosphate buffer) (pH 8.0). After concentration by ultrafiltration through a PM30 membrane (Amicon, Lexington, Mass.), this preparation was further purified on a upward flow 5 x 50 cm G-200 Sephadex column equilibrated and eluted with 0.01 M phosphate buffer-0.2 M NaCl (pH 7.5). Protein, which eluted as a single peak, was pooled and concentrated as described above. This preparation yielded a single band with a potent goat antiserum to human IgG on immunoelectrophoresis. The IgG preparation (2.3 mg and 3.3 mg, respectively) in complete Freund's adjuvant was injected into a burro on two occasions, 1 month apart, and burro serum was collected 3 weeks after the last immunization.

IgG (Fab)<sub>2</sub> was prepared by pepsin digestion (14) of IgG (Cohn fraction II, American Red Cross). The pepsin digest was fractionated by gel filtration through a 4 x 50 cm Sephadex G-200 column equilibrated and eluted with 0.1 M tris-HCl-0.2 M NaCl (pH 7.5). Protein from the first peak was pooled and concentrated as above. Analysis by immunoelectrophoresis with goat anti-IgG (Fc) did not reveal any bands. The IgG (Fab)<sub>2</sub> was covalently linked to S2B (13) and used to absorb the burro anti-IgG. The absorbed burro anti-IgG (Fc) revealed one band on immunoelectrophoresis with an IgG papain digest, and one band was also seen with normal human serum.

**IgE-DARIA.** Total serum IgE was measured by a double-antibody radioimmunoassay (DARIA) as described previously (15), with the modification that the second-stage incubation was shortened to 4 hr at 22°C.

**IgG-DARIA.** Total IgG in sera was measured by an automated nephelometric technique (16, 17). Quantities of IgG not measured by nephelometry (less than 2.94 mg/ml) were measured by a DARIA as described by Miller *et al.*<sup>4</sup> Briefly, the performance of the IgG-DARIA followed the techniques outlined for the IgE-DARIA (15) with the following modifications: 1) specific rabbit antihuman IgG was diluted 1:10,000 with 1% bovine serum albumin (BSA) (Sigma Chemical Co.), 2) 100 µl of <sup>125</sup>I IgG (5 ng/ml) was added in the first stage, and 3) 50 µl of burro anti-rabbit IgG and 50 µl of normal rabbit serum diluted 1:10 with 1% BSA were added in the second stage.

**Preparation of IgG and IgE pools.** IgG protein was purified from human sera by using as starting material clotted plasma from HBV-sensitive patients undergoing venom immunotherapy. Clotted plasma was fractionated by affinity chromatography over a 1.2 x 24 cm S2B anti-IgE column and the column

was washed with 0.01 M phosphate buffer-0.15 M NaCl (pH 7.5). The protein that passed through the anti-IgE column unretarded contained negligible amounts of IgE by DARIA (15) and was further purified by chromatography over a 2.5 x 50 cm column of DEAE Sephadex (Pharmacia Laboratories) equilibrated and eluted with 0.01 M phosphate buffer (pH 7.5). The IgG protein that was not bound by DEAE Sephadex contained in all preparations less than 5 ng IgE/ml by DARIA and, in an analysis for IgE antibodies to PLA by RAST (see below), showed only 0.7% of total radioactive counts bound (comparable with control-serum binding). On immunoelectrophoresis, single bands were seen with a potent antiserum to normal human serum.

IgE protein was purified from clotted plasma from HBV-sensitive patients undergoing venom immunotherapy by two procedures. In the first procedure, IgE was eluted from anti-IgE S2B. In preliminary experiments, IgE (PS) was radiolabeled with <sup>131</sup>I (18) and 34 ng containing 875,000 counts per minute were added to 10 ml of clotted plasma from a control ragweed-sensitive patient containing 11,000 ng/ml of IgE. Between 85% and 94% of total counts added were bound to the S2B anti-IgE columns. The percentages of IgE recovered with several eluting agents were: 19.7% with MgCl<sub>2</sub> (5 M, pH 5.7); 9.2% with glycine-HCl (0.2 M, pH 2.0); 3.4% and 21.6%, respectively, with guanidinium-HCl (1 M and 2 M, pH 4.0); and 54.5% with guanidinium-HCl (4 M, pH 7.0). Guanidinium-HCl (4 M, pH 4.0) was the best reagent for eluting <sup>131</sup>I IgE from S2B anti-IgE (75% of <sup>131</sup>I IgE was recovered) and this reagent was used subsequently in the purification procedure. After dialysis against 0.15 M NaCl the IgE-rich preparations (Table I) contained small amounts of IgG as determined by DARIA and were further purified by affinity chromatography over S2B anti-IgG. By DARIA these preparations were significantly depleted of IgG (less than 1 µg IgG/ml), and in standard radioimmunoassay (RIA) procedures for IgG antibody to PLA, binding comparable with a normal serum control was seen. Recovery of IgE averaged 3% of the original total IgE in the clotted plasma.

In the second procedure IgE protein from one of the venom-treated HBV-sensitive patients was purified without the use of guanidinium-HCl. Clotted plasma was fractionated over a 2.5 x 25 cm column of DEAE-cellulose (DE-52, Whatman Inc., Afton, N. J.) equilibrated with a 0.005 M phosphate buffer (pH 8.0) and eluted with a 0 to 0.5 M linear gradient of NaCl. Protein fractions from the DEAE column containing IgE antibody to PLA by RAST were pooled, concentrated, and gel filtered on a 3.2 x 190 cm Sephadex G-200 column equilibrated and eluted with 0.01 M phosphate buffer-0.2 M NaCl (pH 7.5). Three protein peaks emerged, and fractions containing IgE antibody were pooled and concentrated. To remove IgG and IgA protein, which still contaminated the IgE preparation, three successive immunoabsorptions were performed with 2.5 x 15 cm S2B anti-IgG and S2B anti-IgA columns in tandem equilibrated and eluted with 0.02 M H<sub>3</sub>BO<sub>3</sub>-0.04 M NaOH-0.16 M NaCl (pH 8.0). This second IgE preparation contained, by DARIA, 170 ng IgG/ml and 870 ng IgE/ml. In the RAST for PLA, 19.8% of total radioactive counts were bound, and in a RIP procedure for IgG antibody to PLA, binding comparable with a normal serum control was seen. In this second procedure for purification of IgE, approximately 15% of the total IgE was recovered.

**Analyses of IgG antibody to PLA.** IgG antibody to PLA was measured by a RIP procedure (19). Dilutions of sera or IgG preparation (1:20) were prepared with 0.1 M phosphate buffer (pH 7.5) containing 1% BSA and 0.1% sodium azide. Briefly, 2.5 µg of <sup>131</sup>I PLA (an amount noted to be in marked antigen excess)

<sup>4</sup> Miller, R. D., G. J. Gleich, K. Offord, and S. J. Dunnette. Humoral immune characteristics in chronic obstructive pulmonary disease: A matched pair study. In preparation.

TABLE I

| Patient | Total IgE                | Supernatants                 |                                 |                              |                            |
|---------|--------------------------|------------------------------|---------------------------------|------------------------------|----------------------------|
|         |                          | IgE HSA-S2B abs <sup>a</sup> | IgE Cyto c-S2B abs <sup>a</sup> | IgE PLA-S2B abs <sup>a</sup> | IgE ab to PLA <sup>b</sup> |
|         |                          | ng/ml                        |                                 |                              |                            |
| 1       | 4130 (29.2) <sup>c</sup> | 4493 (34.3)                  | 4235 (32.9)                     | 3054 (2.4)                   | 1232                       |
| 2       | 192 (9.8)                | 194 (10.5)                   | 189 (9.2)                       | 143 (1.1)                    | 49                         |
| 3       | 428 (17.5)               | 456 (17.2)                   | 421 (16.4)                      | 326 (1.3)                    | 109                        |
| Control | 502 (1.0)                | 446 (1.2)                    | 423 (1.0)                       | 456 (0.9)                    | 1                          |

<sup>a</sup> Total IgE in the supernatants after absorption (abs) with HSA, Cyto c, and PLA-S2B.

<sup>b</sup> IgE antibody (ab) to PLA is calculated as the average of (total IgE + IgE HSA-S2B abs + IgE Cyto c-S2B abs) minus IgE PLA-S2B abs.

<sup>c</sup> Percentage of total counts bound in a PLA-RAST is indicated in parentheses for each preparation.

was added to 100  $\mu$ l of the IgG preparation from venom-treated HBV-sensitive individuals or the control pool. After incubation at 37°C for 1 hr, and overnight at 4°C, 0.25 ml of burro antihuman IgG (Fc) was added, and the resulting precipitates formed after a second incubation period (4 hr at 22°C) were washed twice with 0.1 M phosphate buffer (pH 7.5) to remove any unbound <sup>125</sup>I PLA. The radioactivity of the washed precipitates was measured in a gamma scintillation counter, and the results were expressed as nanograms PLA bound per tube. IgG antibody to PLA in nanograms was determined by: 1) subtracting the control binding from the binding of the IgG preparations; 2) dividing by 17,500 (m.w. of PLA) to obtain nanomoles of PLA bound; 3) dividing by 2 (assuming 2 moles of PLA were bound per mole of IgG antibody to PLA); and 4) multiplying by 155,000 (m.w. of IgG).

Two other binding assays were employed in an attempt to determine IgG antibody to PLA. In the first, S2B-PLA was packed into 1 ml tuberculin syringes (Pharmaseal Labs, Glendale, Calif.) with a small quantity of glass wool at the bottom to serve as a filter. Thirty-two to ninety micrograms of radioiodinated <sup>125</sup>I (20) IgG preparation from the venom-treated sensitive individuals or the control pool were added to the columns that were equilibrated and eluted with 1% BSA. Radioactivity (as measured in a gamma scintillation counter) not eluted with 1% BSA (less than 100 cpm) was eluted with 0.05 M glycine-HCl (pH 2.1). Results were expressed as total counts added, and total recovery of counts was consistently 95% to 100% of total counts added. In the second assay, cellulose-PLA (0.1 to 1.0 mg) was combined with the <sup>125</sup>I IgG antibody preparations (0.1 to 5.0  $\mu$ g) in 10 x 75 mm plastic tubes and rotated for 12 hr at 22°C. Excess <sup>125</sup>I IgG was removed by thorough washing as described for the RAST (13) and the results were expressed as percentage of total radioactive counts bound to the polymer.

**Measurement of IgE antibody by the radioallergosorbent test (RAST).** RAST (5) determinations were carried out as previously described (13). Briefly, 500  $\mu$ l of a solid-phase antigen polymer, 1 mg/ml, prepared by linking 10 mg of PLA to 250 mg of cyanogen-bromide activated microcrystalline cellulose (Brinkmann, Westbury, N.Y.), was combined with 50  $\mu$ l of the IgE preparations. After overnight incubation and washing, 100  $\mu$ l (20 ng) of <sup>131</sup>I anti-IgE were added and the mixture was again incubated overnight, washed as before, and counted in a gamma scintillation counter. Results were expressed as percentage of total radioactive counts bound to the cellulose.

**IgG interference in the radioallergosorbent test.** The interference of IgG antibodies in the RAST was tested in the following manner: 1) 50 to 500  $\mu$ l of the IgG preparations were

combined with 20 to 100  $\mu$ l of the IgE preparations, 2) 500  $\mu$ l of PLA cellulose (1 mg/ml) were added, and 3) total volumes were adjusted to 1 ml before the test was completed as described above.

**Measurement of IgE antibodies by radioimmunoprecipitation.** A RIP procedure (21) was adapted to measure the binding of IgE antibody to PLA. Briefly, PLA was radiolabeled with <sup>131</sup>I by the chloramine-T method and after removal of the unreacted <sup>131</sup>I by Sephadex G-25 gel filtration and dialysis with 0.15 M NaCl (13), solutions containing 5 ng/ml to 4680 ng/ml were prepared with 4% FCS (Grand Island Biological Co.) (13). One hundred microliters of purified IgE from either the venom-treated HBV-sensitive individuals or the control pool (both diluted 1:5 with 4% FCS) and 100  $\mu$ l of <sup>131</sup>I PLA (spanning a range of 0.5 ng to 468 ng/tube) were added to 9 x 72 mm conical glass tubes and incubated 1 hr at 37°C and 12 hr at 4°C. After the addition of 100  $\mu$ l of burro antihuman IgE (Fc) (a quantity determined by precipitin analysis to be in antibody excess), 100  $\mu$ l (45  $\mu$ g) of IgE myeloma protein (PS) and 150  $\mu$ l of 4% FCS phosphate buffer were added and the tubes were further incubated at 22°C for 3 to 5 hr. The resulting precipitate was separated by centrifugation and the supernatant was discarded. The precipitate was washed three times with 2.5 ml of an iced 1% tween, 0.1 M phosphate buffer (pH 7.5). After each wash, the precipitate was resuspended and centrifuged. The radioactivity of the washed precipitate was measured in a gamma scintillation counter.

**Measurement of IgE antibodies by immunoabsorption.** In order to measure the amount of IgE antibody to PLA in our IgE preparations, an immunoabsorption procedure was developed (Fig. 1). Human serum albumin (HSA) (crystallized, lyophilized, Sigma Chemical Co.), cytochrome c (Cyto c) (beef heart, Sigma Chemical Co.), and PLA were coupled to S2B as described earlier (13) with the following modification: 50 mg of HSA, Cyto c, or PLA were coupled to 10 ml packed volume of S2B. In each case, greater than 99% binding (as determined by monitoring the spectrophotometric absorbance at 277 nm of the coupling supernatants and washes) was achieved. Two-tenths milliliter to 1 ml of each of the IgE antibody preparations from the three HBV-sensitive patients undergoing immuno-therapy or the control pool were added to 0.5 ml of each of the immunoabsorbents (HSA-S2B, PLA-S2B, and Cyto c-S2B) in 10 x 75 mm plastic tubes (Pharmacia Laboratories). Buffer (1% BSA, 0.1% NaN<sub>3</sub>, 0.1 M phosphate buffer, pH 7.5) was added to each tube to bring the total volume to 2 ml. The tubes were capped and rotated for 24 hr at 22°C and after centrifugation, the total IgE in each supernatant was measured by DARIA (15).

**Determination of the relative avidity of IgG and IgE antibodies to PLA.** IgE and IgG antibodies were tested for their abilities to bind radiolabeled PLA by using limiting quantities of <sup>131</sup>I PLA. In a given experiment, 100  $\mu$ l of IgE antibody or IgG antibody (equimolar quantities as determined by immunoabsorption and IgG-RIP) were combined with 100  $\mu$ l of <sup>131</sup>I PLA (0.1 to 10 ng) in conical glass tubes and incubated at 37°C for 1 hr and 4°C for 12 hr. In the case of IgE protein, 100  $\mu$ l (45  $\mu$ g) of a 1:100 dilution with 4% FCS of an IgE myeloma (PS), 100  $\mu$ l of antihuman IgE, and 150  $\mu$ l of 4% FCS were added to the tubes. In the case of IgG protein, varying amounts of IgG myeloma serum (75 to 100  $\mu$ l), such that the final quantity was approximately 25  $\mu$ g per tube, were added, followed by 250  $\mu$ l of antihuman IgG and a sufficient volume of 4% FCS to adjust the final volume to 550  $\mu$ l. Antihuman IgG and antihuman IgE were added in antibody excess as determined in preliminary

experiments. After 4 hr at 22°C, the precipitates were washed three times with a 0.1 M phosphate buffer (pH 7.5) and the remaining radioactivity was measured in a gamma scintillation counter.

# RESULTS

The quantities of IgE antibody to PLA in the purified IgE protein preparations were determined by immunoabsorption, with HSA-S2B and a basic protein immunosorbent Cyto c-S2B as controls (Fig. 1). The measurement of absolute quantities of IgE antibodies (ab) by immunoabsorption is shown in Table I. As can be seen, no significant decrease in total IgE was seen after absorption of the IgE preparations with either HSA-S2B or Cyto c-S2B and likewise no significant decrease in the reactivity of IgE antibody in the PLA RAST was seen. Absorption of the patient IgE preparations with PLA-S2B revealed substantial reductions in total IgE in all three IgE preparations, and PLA RAST binding decreased to control levels. The control IgE preparation showed no decrease in total IgE with PLA-S2B absorption.

In preliminary experiments we attempted to measure the quantities of IgG antibodies by two direct binding procedures and by the quantitative precipitin method (22). Initially we radiolabeled the purified IgG protein containing antibody to PLA and attempted to determine the number of counts bound to a small volume of S2B-PLA in a tuberculin syringe; however, the quantities of counts bound and eluted were not large enough to discriminate the labeled IgG antibody and the labeled IgG control. Subsequently, we exposed the radiolabeled IgG antibody to PLA-cellulose in tubes, but again we could not consistently discriminate the quantity of counts bound to the PLA-cellulose by the test and control IgG preparations. Next we tried to measure the IgG antibody by the quantitative precipitin reaction. Although faint visible precipitates formed in the patients' serum samples, the quantities were so small that they were lost during the washing procedure. Finally, we elected to measure IgG antibody by the RIP procedure as described in *Materials and Methods*.

Results of the measurement of percentage of IgE and IgG antibodies to PLA in three HBV-sensitive patients undergoing HBV immunotherapy are presented in Table II. IgE antibody as determined by the immunoabsorption procedure varied from 25.1% to 28.7% of total IgE and was similar in all three patients

TABLE II

| Patient | Total IgE <sup>a</sup> | IgE ab <sup>a</sup> | IgE ab | Total IgG <sup>b</sup> | IgG ab <sup>c</sup> | IgG ab |
|---------|------------------------|---------------------|--------|------------------------|---------------------|--------|
|         | ng/ml                  |                     | %      | mg/ml                  | μg/ml               | %      |
| 1       | 4130                   | 1232                | 28.7   | 13.37                  | 9.77                | 0.073  |
| 2       | 182                    | 49                  | 25.5   | 12.71                  | 27.02               | 0.210  |
| 3       | 428                    | 109                 | 25.1   | 10.62                  | 10.14               | 0.095  |

<sup>a</sup> Total IgE and IgE antibodies (ab) from Table I.

<sup>b</sup> Total IgG was measured by nephelometry and/or DARIA.

<sup>c</sup> IgG ab was determined by radioimmunoprecipitation.

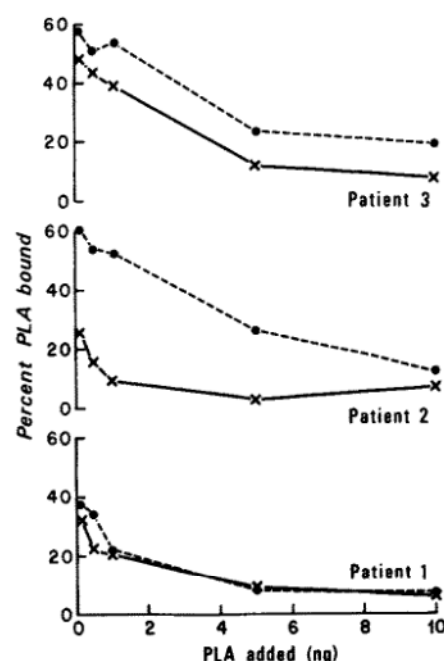


Figure 2. Comparative avidity of IgE antibody and IgG antibody for <sup>125</sup>I PLA. The avidity of equimolar concentrations (patient 1,  $3.08 \times 10^{-14}$ ; patient 2,  $2.44 \times 10^{-14}$ ; patient 3,  $5.45 \times 10^{-14}$  moles) of IgG antibody (●—●) and IgE antibody (x—x) for <sup>125</sup>I PLA in three patients is represented as the percentage of PLA bound in separate RIP procedures for IgE and IgG.

despite the wide range of total IgE. IgG antibody to PLA was measured by the RIP procedure and varied from 0.073% to 0.210% of total IgG (Table II).

The comparative avidities of the IgE and IgG antibody preparations for PLA were measured by RIP with a range of 0.1 ng to 10 ng <sup>125</sup>I PLA. IgG and IgE preparations from a given patient were tested in the same assay. In Figure 2, the percentages of bound <sup>125</sup>I PLA at various concentrations of <sup>125</sup>I PLA are shown for equimolar concentrations (patient 1,  $3.08 \times 10^{-14}$ ; patient 2,  $2.44 \times 10^{-14}$ ; patient 3,  $5.45 \times 10^{-14}$  moles) of IgE and IgG antibody. In one patient, the avidities of PLA for IgE and IgG antibodies were essentially identical (patient 1). In another patient (patient 3), IgG antibody bound PLA slightly more avidly than IgE antibody. In the final patient (patient 2), the avidity of IgG antibody was greater than IgE antibody for PLA. At 10 ng of added <sup>125</sup>I PLA, binding of IgE and IgG antibodies were indistinguishable from control binding in all three patients.

Because the avidity of the IgE antibody preparation might have been affected by the isolation procedure (i.e., elution from S2B anti-IgE with 4 M guanidinium-HCl), a second IgE preparation was isolated without the use of guanidinium-HCl. Two 1-ml aliquots of the second IgE preparation—one treated with

## Measurement of IgE Antibody to Phospholipase-A by Immunoabsorption

IgE preparation absorbed with:

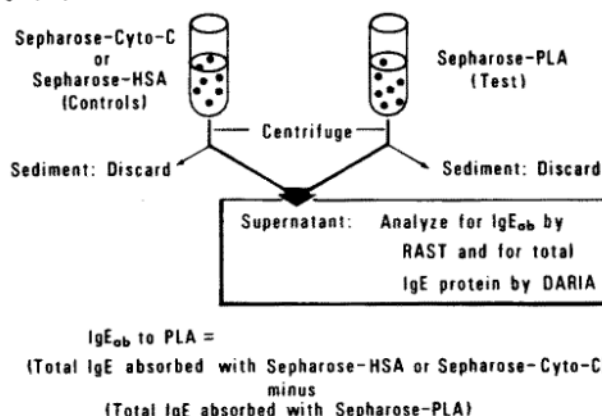


Figure 1. Measurement of IgE antibody (ab) to PLA by immunoabsorption.

4 M guanidinium and one untreated— were compared in a binding experiment with PLA (Fig. 3). After 3 to 4 hr the guanidinium-HCl was removed from the former preparation by gel filtration through Sephadex G-25, and we found that a 50% loss of total IgE as measured by DARIA occurred. Therefore, in the experiment the total IgE concentrations in the two preparations were adjusted so that they were equal. As seen in Figure 3, the avidity of the guanidinium-HCl-treated IgE preparation did not differ significantly from the avidity of the untreated IgE preparation, suggesting that the 4 M guanidinium-HCl treatment did not affect the binding of IgE antibody to PLA.

With purified IgE protein, we next estimated the valence of IgE antibody by determination of the binding of PLA (Fig. 4). As an increasing quantity of PLA was added, the amount of free PLA increased and  $r/c$  decreased toward 0 (zero) while  $r$  (the number of moles PLA bound per mole of IgE antibody to PLA) approached 2.

Finally we tested whether IgG antibody to PLA could inhibit the binding of IgE antibody to PLA in the RAST. The results are shown in Figure 5. IgG antibody was able to inhibit the

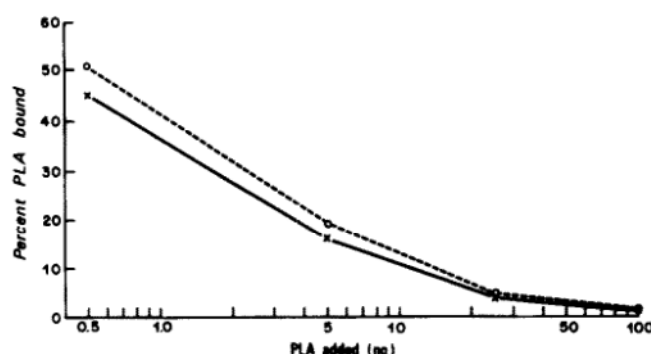


Figure 3. Effect of 4 M guanidinium-HCl on the avidity of IgE antibody for  $^{125}\text{I}$  PLA. Comparison of the avidity of an IgE preparation from a HBV-sensitive individual using (1) IgE preparation treated with 4 M guanidinium hydrochloride ( $\times$ — $\times$ ) and (2) IgE preparation untreated with 4 M guanidinium-HCl ( $\circ$ — $\circ$ ).

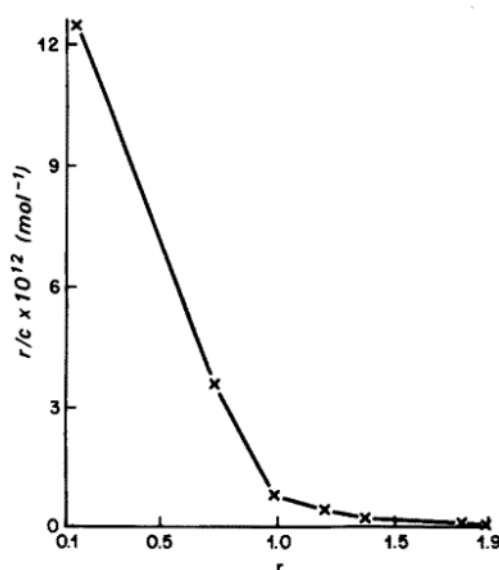


Figure 4. Valence of IgE antibody to PLA as measured by an IgE radioimmunoabsorption procedure.  $r$  = moles of PLA bound/mole of IgE antibody to PLA;  $1/c$  = 1/mole PLA free (see text).

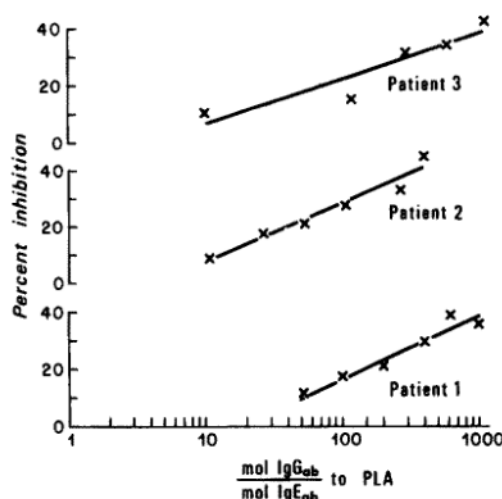


Figure 5. RAST inhibition of the binding of IgE antibody (ab) to PLA by IgG antibody (ab). Correlation coefficients for least squares regression lines were patient 1,  $r = +0.966$ ; patient 2,  $r = +0.972$ ; patient 3,  $r = +0.928$ .

binding of IgE antibody in the RAST from 10% to 40% at a molar excess of 10- to 1000-fold. In patient 2, IgG antibody was considerably more avid than IgE antibody (Fig. 2) and at a 400 M excess of IgG antibody, a 40% decrease in the binding of IgE antibody in the RAST was seen. The IgG antibody of patient 3 was moderately greater in avidity than the IgE antibody and a 1100-fold molar excess of IgG antibody produced a 42% decrease in the binding of IgE antibody in the RAST. The IgG and IgE antibodies of patient 1 had similar avidities for PLA, and a 1000-fold molar excess of IgG antibody produced a 36% decrease in the binding of IgE antibody in the RAST.

## DISCUSSION

In this paper we describe a method for isolation of IgE and IgG proteins and the results of tests of these proteins for antibody activity to a purified allergen, PLA. IgE and IgG-rich fractions, depleted of other immunoglobulins, were prepared by affinity chromatography, gel filtration, and ion-exchange chromatography of clotted plasma from venom-treated HBV-sensitive individuals. Eighty percent of total IgG was recovered, whereas only an average of 3% of total IgE was recovered. The IgG preparations contained insignificant amounts of IgE antibody to PLA judged by RAST, and by DARIA small amounts of IgE protein were detected. The IgE preparations contained insignificant amounts of IgG antibody to PLA by RIP and by DARIA small amounts of total IgG were found.

In immunoabsorption experiments conducted with the IgE preparations in three venom-treated HBV-sensitized individuals, IgE antibody accounted for 25.1% to 28.7% ( $\bar{X} = 27.0\%$ ) of the total IgE. In a normal serum control IgE preparation, no IgE antibody to PLA was found. These percentages are similar to those reported for ragweed and grass pollen antigens by Gleich and Jacob (1), for benzylpenicilloyl determinant and ragweed antigen E reported by Schellenberg and Adkinson (2), and for ragweed antigen E by Zeiss *et al.* (3). In the RIP procedure with the IgG preparation from three HBV-sensitive venom-treated individuals, IgG antibody to PLA accounted for 0.073% to 0.210% ( $\bar{X} = 0.130\%$ ) of total IgG. These results are consistent with the low levels of IgG and IgA antibodies (less than 2%) which were found by Bandilla and associates (23) after immunizing humans with hemocyanin.

The avidities of the IgE and IgG antibodies to PLA were tested in the three patients. In one patient, equimolar quantities of IgE and IgG antibody bound equivalent amounts of PLA whereas in another patient IgG antibody bound slightly more avidly to PLA than IgE antibody. IgG antibody was significantly more avid for PLA than IgE antibody in the third patient. The patient with the greatest absolute amount of IgG antibody also had the most avid IgG whereas the patient with the least IgG antibody to PLA had the least avid IgG.

Because the IgE preparations were subjected to harsh physical conditions, namely exposure to 4 M guanidinium-HCl, we were concerned that this treatment may have affected the antigen-binding site or the conformation of the Fab portion of the molecule. For this reason, a second IgE antibody preparation was prepared without the use of anti-IgE affinity chromatography. Equal quantities of this preparation when mixed with 4 M guanidinium-HCl or left untreated displayed almost identical avidities for PLA. This result suggests that 4 M guanidinium-HCl did not appreciably alter the PLA binding sites on IgE antibody in this preparation and encourages belief that the soluble protein in the IgE preparations was not altered in its avidity.

Ishizaka and Ishizaka (24) suspected that IgE was a divalent antibody as it was able to agglutinate antigen E-coated red blood cells. They showed that IgE and IgG had comparable agglutination that could be inhibited by addition of excess antigen E. Likewise, our studies of the valence of the IgE antibody to PLA suggest that the valence is two.

The inhibition of the binding of IgE antibody to a solid phase allergen by IgG antibody was mentioned by Schellenberg and Adkinson (2); however, in their study this possibility was not tested. Vervloet and associates (25) demonstrated the presence of blocking factors in the serum of patients with bronchopulmonary aspergillosis which could partially inhibit the RAST to *Aspergillus fumigatus*. Lynch and associates (26) likewise demonstrated that IgG antibody to *Alternaria* could competitively inhibit the *Alternaria*-cellulose RAST in children with chronic asthma. Our data indicate that IgG antibody to PLA in a molar excess from 10- to 1000-fold inhibits the binding of the IgE antibody to PLA in the RAST from 10% to 40%. In addition, patient 2, who had the most avid IgG antibody, inhibited most strongly in the RAST, whereas patient 1, who had the least avid IgG antibody, inhibited least strongly in the RAST.

Clinical immunity to honeybee stings in some HBV-sensitive individuals seems to be correlated to the level of IgG blocking antibody to PLA (9, 10). Knowledge of the absolute amounts of IgG and IgE antibody and the interaction of these two antibodies with PLA is not only necessary for comprehending the results of immunotherapy but also for designing more effective programs of immunotherapy. The results of the RAST inhibition experiments have broad clinical significance. Spurious low values for IgE antibody as measured by RAST may be related to the development of high levels of IgG blocking antibody. For example, the decline in IgE antibody that has been reported in some studies after ragweed hyposensitization (27) may be artifactual due to the increased levels of IgG antibody and the inhibition of RAST binding; however, in our laboratory we have not been able to demonstrate that IgG antibodies to ragweed interfere with the binding of IgE antibodies to ragweed in the RAST (28). Further studies of the relationship of IgE and IgG antibodies must be conducted before generalization to other allergen systems. The properties of IgG blocking antibody and its interaction with IgE antibody

are integral to understanding modifications of the allergic response produced by immunotherapy to venom antigens.

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