

MEASUREMENT OF IgE ANTIBODY BY AN ANTIGEN-BINDING ASSAY: CORRELATION WITH PK ACTIVITY AND IgG AND IgA ANTIBODIES TO ALLERGENS¹

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A quantitative radioimmunoassay for IgE antibodies (ab) is described. The assay measures IgE antigen-binding activity (BA) by using purified allergens radiolabeled with ¹²⁵I, IgE myeloma serum as a source of carrier protein, and monospecific goat anti-IgE (anti-IgE) to precipitate the IgE. IgE BA for the Group I protein of rye grass pollen (Rye I) was measured in the serum of patients with grass pollen hay fever who had never received immunotherapy. Values for IgE BA were found to correlate well with radioallergosorbent technique (RAST) results ($r = 0.89$, $p < 0.001$). By assaying IgE-rich fractions, pure IgG, and whole serum, we found a good correlation between IgE BA, Prausnitz-Küstner (PK) activity, and total IgE. A positive PK test could be elicited with 0.05 ml of dilutions containing as little as 1 or 2 units IgE BA for Rye I/ml. PK testing was more sensitive than either RAST or the antigen-binding assay. Estimates based on the quantity of antigen bound in antigen excess suggested that 1 unit IgE BA represented 0.1 ng IgE ab. With higher concentrations of antigen (up to 1 μ g/ml), however, estimates suggested that 1 unit IgE BA represented as much as 0.4 ng IgE ab. These higher estimates appeared to correlate reasonably well with estimates based on RAST elution or RAST absorption. The assay for IgE BA was directly comparable with assays for IgG BA and IgA BA. Results of these assays on sera from the patients showed that IgG BA for Rye I was detectable in 25 of 25 IgE BA in 24 of 25, and IgA BA in 21 of 25. Quantitatively we found a characteristic pattern IgG BA > IgE BA > IgA BA (geometric mean values 862, 214, and 60.2 units/ml, respectively). In keeping with this there was a highly significant quantitative correlation between IgG BA and IgE BA ($r = 0.72$, $p < 0.001$). None of the sera from nonallergic persons contained detectable IgA or IgE BA for Rye I; however, 20% of these sera (10 of 48) contained IgG BA.

The studies that established that IgE antibody (ab) was responsible for reaginic activity in human serum (1) also confirmed the presence of IgG and IgA ab in those sera (2). Antigen-binding assays at that time and subsequently have suggested that IgE ab represented a significant proportion of the total serum IgE (1, 3). Because of the low levels of serum IgE, however, these assays were complicated and not directly comparable with assays for other classes (1, 3). Since the introduction of the radioallergosorbent technique (RAST)³ (4), this has become the method of choice for detecting IgE ab in the serum of patients. RAST, as routinely used, is not quantitative. Two recent reports, however, have shown that absolute measurements of the IgE attaching to RAST beads can be made. Schellenberg and Adkinson (5) assayed IgE released from RAST beads at alkaline pH and found on average that 6% of the total IgE in the serum of patients with hay fever was specific ab to ragweed antigen E (antigen E). They also showed that quantitative values could be obtained for RAST results by use of a control serum whose IgE ab had been measured by RAST elution (5). Gleich and Jacob (6) measured total IgE in the serum of hay fever patients before and after absorption and found that 30% of the total IgE could be specifically removed by pollen antigens linked to Sepharose beads.

To compare results for different classes we were interested in developing an assay for IgE ab that could be applied to IgG, IgA, and IgE in parallel. We also wanted an assay that would be sufficiently sensitive to measure ab in secretions or in serum from nonallergic persons. The present report describes the use of an antigen-binding assay for IgE ab by using myeloma serum as a source of carrier protein, in parallel with assays for other classes. The assay has been compared with RAST and PK activity. Estimates of the quantity of IgE ab made from IgG and IgE-binding activity have been compared with total IgE and parallel estimates based on RAST absorption (6) and RAST elution (5).

MATERIALS AND METHODS

Monospecific antisera. The preparation of monospecific antisera has been described previously (7-9). Briefly, a goat was immunized with the Fc fragment of E myeloma protein, P.S., in complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich.). The anti-IgE was made monospecific by repeated

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³ Abbreviations used in this paper: Anti-IgE, Anti-IgA, and Anti-IgG, goat or rabbit anti-sera specific for heavy chain determinants of human IgE, IgA, and IgG; BBS, borate-buffered saline, pH 8.0; Rye I, the group I protein from rye grass pollen; Antigen E, antigen E of ragweed pollen; BA, binding activity; RAST, radioallergosorbent technique; PK, Prausnitz-Küstner; SAS, saturated ammonium sulfate; HSA, human serum albumin; G.M., geometric mean.

passages over an IgG immunosorbent column. In addition the goat anti-IgE was specifically purified by elution from an IgE immunosorbent column with glycine buffer, pH 2.8 (7, 10). Rabbits were immunized with human IgA or the Fc fragment of human IgG in CFA. The anti-IgA was made monospecific by passage over an IgG immunosorbent column and a column of IgA-deficient serum linked to immunosorbent. This IgA-deficient serum contained normal quantities of IgG, IgM, and IgE but <10 ng/ml IgA as measured by radioimmunoassay (9). The anti-IgG was made monospecific by repeated passage over an Fab immunosorbent column and also over an IgE immunosorbent column. IgE, IgG, the Fab fragment of IgG, and IgA-deficient serum were linked to Sepharose 2B by the cyanogen bromide technique (10).

Antigens. Highly purified Group I protein from rye grass pollen (Rye I) was kindly donated by Dr. David Marsh, the Johns Hopkins University (11). Forty micrograms of Rye I was labeled with 2 mCi ^{125}I by the chloramine-T technique (12). After 2 days of dialysis, 84% of the radioactivity remaining could be precipitated by trichloroacetic acid. Sixty-two percent of the radioactivity could be bound by IgG ab in a human serum, but this is probably a minimum estimate because the ab was not in great excess and the antigen concentration used was low. In calculating the specific activity of the Rye I, the quantities of Rye I bound, and the antigen excess, we assumed that 84% of the radioactivity was bound to Rye I. The specific activity of the ^{125}I -labeled Rye I was 29,400 cpm/ng (i.e., 84% of 35,000 cpm/ng added). Lyophilized aqueous extract of Rye grass pollen was kindly provided by Dr. David Moran (Bencard Ltd., England). This extract was dissolved in borate-buffered saline, pH 8.0 (BBS), centrifuged at $3000 \times G$ for 1 hr, and passed over a 75- x 2.5-cm Sephadex G-100 column. Dilutions of the eluted fractions were assayed for their ability to inhibit the binding of ^{125}I Rye I by IgG ab in the control serum used for the assay (see below). The fractions rich in Rye I were pooled, concentrated, and dialyzed. This pool is referred to as Rye I-rich pool. The concentration of Rye I in this fraction estimated by comparing its ability to inhibit the binding of ^{125}I Rye I with that of cold purified Rye I was approximately 4 mg/ml and this represented approximately 30% of the total protein present. Ragweed antigen E research reagent was obtained from the National Institutes of Health, Bethesda, Md. and was labeled with ^{125}I by a modification of the technique of Klinman and Taylor (8, 12). Short ragweed extract containing 40,000 p.n.u./ml was obtained from Center Laboratories Inc., Port Washington, N. Y.

Patients. Serum was obtained from 40 unselected patients referred to two hospital clinics between October and December 1975 and 1976 for treatment of grass pollen hay fever. The diagnosis was made by clinical history and prick test with grass pollen extract. None of these patients had received immunotherapy in the past. Fifteen patients received immunotherapy with 9 to 11 graded injections of alum-precipitated pyridine extract of whole grass pollen ("Allpyral", Dome Laboratories Ltd., England) (13). Treatment extended over approximately 12 weeks and post-treatment sera were obtained in April. Sera were also obtained from patients with ragweed or grass pollen hay fever who had received immunotherapy with aqueous extracts of pollen. Nonallergic controls were laboratory personnel, patients with chronic urticaria, and patients with nonseasonal rhinitis. All had negative skin tests with grass pollen extract.

Fractionation of sera. Sera were fractionated with saturated ammonium sulfate (SAS) (14) by adding $\frac{1}{2}$ volume of SAS to 1 volume of serum. After 1 hr at room temperature they were

centrifuged at $1,000 \times G$ for 20 min and the supernatant was transferred to a new tube. A further $\frac{1}{2}$ volume of SAS was added to the supernatant giving a final concentration of 50% SAS. The 33 and 33 to 50% precipitates were dissolved in BBS and made up to $2\frac{1}{2}$ times the original volume of serum. For three patients 40 ml of serum were fractionated with SAS and the fractions were further separated. The 33% SAS precipitate was dissolved in and dialyzed against 0.005 M phosphate buffer, pH 8.0, and passed through a DEAE cellulose column (2 x 24 cm) equilibrated with the same buffer. The protein not retarded by the DEAE was pure IgG as judged by immunodiffusion and radioimmunoassay for IgA, IgE, and IgM. The 33 to 50% SAS precipitate was dissolved in and dialyzed against BBS and 5-ml samples were applied to an upward flowing, gravity fed 2.5 x 175 cm Sephadex G-200 (Pharmacia, Uppsala, Sweden) column. The eluate from the column was collected in 6-ml volumes and assayed for IgE and IgG BA for Rye I. Fractions were then pooled, concentrated, and dialyzed against BBS, and are referred to as Fractions I, II, and III.

Double antibody-antigen binding radioimmunoassay for IgE ab to Rye I. This assay was developed from the assay previously used for IgG, IgA, and IgM ab to diphtheria toxin (9, 15) and antigen E (8). To carry out double antibody assays it is necessary to have a quantity of immunoglobulin sufficient to make an adequate precipitate; in general between 10 and 50 μg protein/tube will give reproducible results. Preliminary experiments with two IgE myeloma sera (N.D. and P.S.) showed that either would give a precipitate with anti-IgE in the presence of 3 ng ^{125}I Rye I (or ^{125}I antigen E) which when washed would bind less than 0.2% of the radioactivity added. One-tenth milliliter of a 1/100 dilution of myeloma serum P.S. (total IgE 19.5 mg/ml) was used routinely, and the optimum quantity of anti-IgE was determined by qualitative determination of the equivalence zone (16). Three-tenths percent bovine serum albumin in BBS was used as diluent in all assays. The assay was carried out as shown in Figure I. A suitable dilution of serum or a fraction of serum was mixed with carrier IgE and radiolabeled antigen and allowed to bind for 4 hr at room temperature. For estimates of antigen-binding capacity this incubation time was extended to 8 hr (Table I). Anti-IgE was added and the precipitate was allowed to form at 4°C overnight. The precipitates were washed three times before being transferred to a new tube and washed once again. Radioactivity was counted in the precipitates. IgG and IgA ab to Rye I (or antigen E) were assayed in parallel to IgE ab by using the same quantity of antigen and the same volumes as for the IgE ab assay. The quantity of total IgG and IgA in sera was measured by radial immunodiffusion. Where necessary serum from a nonallergic person was used as a source of carrier IgG or IgA (8).

The positive control serum for the anti-Rye I assays was obtained from a patient who had been repeatedly immunized with aqueous extract of grass pollen and whose pooled serum was arbitrarily defined as containing 40,000 units of IgG BA for Rye I/ml. An IgG BA control curve was always carried out in parallel with the assays by using 2-fold dilutions of this control serum (1/50 to 1/25,600). The control curve was plotted on a log linear scale (see Fig. 4) and values for IgE, IgA, and IgG BA in units/ml were obtained by reference to this curve. Assays were routinely carried out at two dilutions for each class of ab and the results were taken as the mean of the two determinations provided the two results were $\leq \pm 9\%$ from the mean. At least two negative sera were included in each assay. In a separate experiment these negative sera were compared with IgG and IgA myeloma sera, with IgE myeloma serum alone, and with serum from two allergic patients (total IgE 12,000 and

0.1 ml serum or serum dilution
 +
 0.1 ml P.S. serum diluted 1:100
 +
 0.05 ml ^{125}I Rye I or antigen E (60 ng/ml)
 shake
 4 hours at room temperature
 +
 0.1 ml goat anti-IgE
 overnight 4°C
 Wash three times, transfer to a new tube, wash again and count
 radioactivity in the precipitate

Figure 1. Double antibody antigen-binding radioimmunoassay for IgE ab.

3,600 ng/ml) who gave negative skin tests when tested with grass pollen extract. The background for IgG and IgA precipitates was generally very similar to that for IgE. A significant result was defined as 2 standard deviations above background. The variation between repeated determinations of IgE BA in a single experiment was $\pm 6\%$ and between separate experiments was $\pm 9\%$; this variation was very similar to that seen in repeated assays of IgG or IgA BA.

Antigen concentration in the antigen-binding assay. Antigen binding by ab in the fluid phase is dependent on a variety of factors including the degree of antigen excess over ab and the absolute concentration of antigen present (17-19). Consistent results could be obtained for IgG BA with as much as 35% of the added antigen bound (data not shown). However, a linear relationship between the quantity of serum added and the quantity of antigen bound (on a log log plot) was only obtained when $<8\%$ of the added ^{125}I Rye I was bound. In preliminary experiments quantities of ^{125}I Rye I from 0.375 ng to 1.2 μg were added to the assays (data not shown). Three sera were assayed with 108, 18, and 3 ng ^{125}I Rye I and the results were calculated both as nanograms Rye I bound per milliliter of serum and as percentage of added Rye I bound (Table I). Similar results were obtained with three other patients and four nonallergic persons. The results showed first that the relationship between Rye I binding by different classes of ab is similar over a wide range of antigen concentrations, and second that more Rye was bound in the presence of higher concentrations of antigen. One-tenth milliliter of a 1/50 dilution of serum B.L. assayed with 3 ng of Rye I bound 0.046 ng as IgG and 0.018 ng as IgE, which represents 2% of the added Rye I. Nonetheless, the same dilution assayed in the presence of 108 ng Rye I bound 0.09 ng as IgG and 0.046 ng as IgE (Table I). Thus, when comparing tubes with antigen excess as high as 50-fold there is still a marked increase in antigen bound with increasing concentrations of antigen. For different sera the increase in antigen bound varied from 2.5- to 4-fold over the range 3 ng to 0.3 μg Rye I added. Thus, the relationship between BA results for different sera would be altered slightly if more Rye I were added to the assays. Third, the background radioactivity is closely related to the quantity of antigen added, increasing 24-fold with a 36-fold increase in antigen added. The sensitivity of the assay is largely dependent on the background radioactivity, thus, with 3 ng of Rye I added 1.6 units of BA gave a result significantly above background, whereas with 1.2 μg of Rye I added 50 units of BA did not increase the radioactivity in the precipitate above background (data not shown). The assay was routinely carried out with 3 ng of antigen added to each tube.

PK testing. Sera or fractions of sera used for PK testing were tested for Australia antigen, diluted with 0.05% human serum albumin (HSA) in BBS and sterilized by passage through a

Swinnex-13 filter (pore size 0.45 μm , Millipore Corporation, Bedford, Mass.). Three volunteers who had been informed about the risks involved were used for PK testing. Five-hundredths-milliliter aliquots of serial 2-fold dilutions of sera were injected intradermally; after 24 hr the sites were challenged with Rye I (0.05 ml of 0.1 $\mu\text{g}/\text{ml}$) or Ragweed extract (0.05 ml of a 1/1000 dilution). PK tests were read 15 min after challenge and a wheal 6 mm in diameter was regarded as positive.

Radioimmunoassay for total IgE. The double antibody inhibition radioimmunoassay for IgE has been described previously (20, 21). The rabbit anti-IgE was raised by immunizing with IgE myeloma protein (P.S.) (Behringwerke, Germany) and was made monospecific by passage over an IgG immunosorbent column. For radiolabeled antigen we used IgE myeloma protein from a different patient (N.D.) kindly provided by Dr. S. G. O. Johansson, Uppsala, Sweden. The precipitating antibody was obtained by immunizing goats with normal rabbit IgG in CFA. The serum used for the standard curve which contained 5000 IU IgE/ml had been substandardized from the new reference standard 75/502, provided by the National Institute for Biological Standards and Control, London. One IU IgE was regarded as equivalent to 2.4 ng (22).

RAST, RAST elution, and RAST absorption. For RAST (4) Rye I-rich pool containing 10 mg Rye I, or 10 mg HSA were linked to 15 ml Sepharose 4B which had been activated with cyanogen bromide (10). These immunosorbents were washed with acid and alkaline buffers and diluted to 2% in RAST buffer; 0.5 ml was used for each assay. RAST results were standardized by reference to the results with serial dilutions of an IgE-rich fraction of serum (see HB fraction II, Tables III and IV). This fraction contained 1730 units IgE BA for Rye I/ml and was arbitrarily defined as containing 1000 RAST units/ml against Rye I-rich pool. RAST absorption was carried out as described by Gleich and Jacob (6). Briefly, 0.1 ml of serum or a dilution was rotated with rye grass RAST beads or HSA RAST beads. After 24 hr the beads were removed by centrifugation and total IgE was assayed in the supernatant. The results were calculated from the equation:

$$\% \text{ Specific IgE} = \frac{(\text{IgE in HSA absorbed supernatant-IgE in Rye grass absorbed supernatant})}{\text{IgE in HSA absorbed supernatant}} \times 100$$

RAST absorption was carried out in duplicate at two dilutions, generally 1/2 and 1/4. RAST and RAST elution was carried out and calculated exactly as described by Schellenberg and Adkinson (5). Briefly, the rye grass immunosorbent was incubated with serum and then exposed to buffer at pH 10.5 or 7.5. These supernatants were neutralized and assayed for total IgE. The diluted immunosorbents were then washed and incubated with ^{125}I goat anti-IgE as for the direct RAST test. Total IgE was assayed in the supernatants by double antibody inhibition radioimmunoassay (20, 21).

Estimates of the absolute quantities of anti-Rye I ab from its antigen-binding activity. The quantity of IgG anti-Rye I in the control serum was estimated from its antigen-binding activity (18). The amount of Rye I bound by three dilutions within the linear section of the control curve (i.e., with less than 8% of the Rye I added bound) was calculated as shown in Table I. At marked antigen excess, i.e., >10 -fold, it is likely that the majority of ab molecules are in the state Ab antigen₂ (17, 18). Knowing the m.w. of Rye I (27,500 daltons) we calculated on the basis of three separate experiments that with 3 ng Rye I added to the assays 1 unit of IgG BA corresponded to 0.085 ng IgG ab and that 1 unit IgE BA represented 0.1 ng IgE ab. However, as shown in Table I, with higher amounts of antigen added the

TABLE I
Antigen bound by IgG and IgE antibody at different concentrations of Rye I

Serum	Dilutions Assayed	Class of BA ^a	¹²⁵ I Rye I Added to Assays ng ^b						Differ- ence ^c
			108 (432 ng/ml)		18 (72 ng/ml)		3 (12 ng/ml)		
			ng bound/ml ^d	% bound ^e	ng bound/ml	% bound	ng bound/ml	% bound	
L.E.	1/25 and 1/50	IgG	89	(0.51%)	40	(1.45%)	26	(5.6%)	3.4:1
		IgA	26		13.1		8.2		3.17:1
		IgE	23.5		11.6		7.9		3.0:1
		Ratio IgG:IgE ^f	3.8:1		3.4:1		3.3:1		
B.L.	1/25 and 1/50	IgG	45	(0.3%)	38	(1.3%)	20	(3.9%)	2.2:1
		IgA	<8.5		<1.0		<0.4		
		IgE	23		19		9.5		2.4:1
		Ratio IgG:IgE	1.9:1		2:1		2.1:1		
R.H. ^g Control serum	1/400, 1/1600 and 1/6400	IgG	2240	(0.5%)	1470	(2.1%)	980	(8%)	2.3:1
Negative ^h control			0.12 ± 0.022%		0.16 ± 0.03%		0.18 ± 0.03%		24:1

^a Precipitate formed by addition of anti-IgG, anti-IgA or anti-IgE.

^b Nanograms of Rye I added in 0.05 ml, values in parentheses represent the concentration of antigen in 0.25 ml.

^c Rye I bound with 108 ng added:Rye I bound with 3 ng added.

^d Rye I bound, ng/ml of serum = $10 \times \text{serum dilution} \times \frac{(\text{Radioactivity in ppt.} - \text{Radioactivity in control ppt.})}{\text{Specific activity of Rye I}}$

Values are the mean of duplicates at each dilution.

^e % of added Rye I bound by IgG, IgA, and IgE ab at the lowest dilution of serum assayed.

% Rye I bound = $\frac{(\text{Radioactivity in ppt.} - \text{Radioactivity in control ppt.})}{84\% \text{ of Radioactivity added to assay}}$

^f Rye I bound by IgG ab:Rye I bound by IgE ab.

^g When precipitated with anti-IgA or anti-IgE the control serum bound less than 10 ng Rye I/ml with 3 ng added.

^h The negative control value is the mean ± S.D. of 12 tubes (four tubes for each class of ab) containing serum from nonallergic persons or myeloma patients.

sera will bind more antigen. With 108 ng Rye I added the estimates of ab were 2.3-fold higher.

RESULTS

IgE BA in serum and fractions of serum. IgE ab and total IgE were assayed in the sera of 30 unselected patients who had presented for treatment of grass pollen hay fever. IgE ab to Rye I-rich pool in units/ml was assayed by RAST and IgE BA for Rye I was determined by using the antigen-binding assay; for each technique values were extrapolated from a control curve. The results in Figure 2 show a good correlation between the results of the two techniques ($r_s = 0.89$, $p < 0.001$). One serum was negative by both techniques (<3 units IgE BA and <4 units RAST/ml). Six sera from nonallergic persons contained no detectable IgE ab whereas serum from an allergic person who had a total serum IgE of 12,500 ng/ml but no hay fever and negative skin tests to grass pollen gave a weakly positive RAST (16 units/ml) but had no detectable IgE BA. We also found that both IgE myeloma sera gave positive RAST tests at a dilution of 1/1000 but had no detectable IgE BA. Comparing total IgE and IgE BA in the sera we found a significant direct correlation ($r_s = 0.601$, $p = 0.001$) (data not shown). To check the specificity of the antigen-binding assay 10 sera with >200 units/ml of IgE BA for Rye I were assayed for IgE BA for antigen E and none was detected.

Carson *et al.* (14) have reported that human IgE fails to precipitate in 33% SAS but is almost completely precipitated in 50% SAS. The results in Table II show that simple fractionation with SAS can achieve a 6-fold enrichment of IgE BA relative to IgG BA. Furthermore, with some sera from patients who had received immunotherapy and had high levels of IgG BA, IgE BA was detectable in the 33 to 50% SAS fraction but not in whole serum (Table II). Three sera were fractionated more extensively. The 33 to 50% SAS fraction was passed over a Sephadex G-200 column and BA was assayed in the eluted fractions (Fig. 3). The IgE BA-rich fractions eluting before the IgG BA were pooled as Fraction II. The protein precipitated by 33% SAS was passed over DEAE equilibrated with 0.005 M phosphate buffer, pH 8.0, and the protein not retarded by DEAE was taken as pure IgG. Total IgE and BA were then assayed in whole serum, the IgG fractions and the IgE-rich fractions from these three sera (Table III). The results show a very marked enrichment of IgE BA relative to IgG BA in Fraction II; for one serum (L.P.G.) the enrichment was better than 100-fold, so that more than 90% of the BA in Fraction II was IgE. The relationship between IgE BA and total IgE in the IgE rich fractions was in each case similar to that in whole serum. One of the sera analyzed was from a patient who had received immunotherapy and despite an enrichment of IgE BA of almost 30-fold, Fraction II still contained more IgG BA than IgE BA. However, despite the large excess of IgG BA in this

serum (H.B.) the value obtained for IgE BA in whole serum, 900 units/ml, compares closely with the value of 1730 units/ml found in Fraction II when corrected for the levels of total IgE, 1010 and 2050 ng/ml, respectively. The results for the IgG fraction of this serum showed no significant IgE BA detectable even with a 250-fold excess of IgE BA.

In these experiments we have used a control curve obtained by assaying IgG BA in serial dilutions of a serum containing high levels of IgG ab. In using this curve to quantitate IgE BA we have assumed that serial dilutions of IgE ab would give a curve of the same shape. The results in Figure 4 show that the curve for IgE BA in serial dilutions of Fraction II (N.L.) was superimposable over the IgG BA control curve. This was confirmed with Fraction II from the other sera.

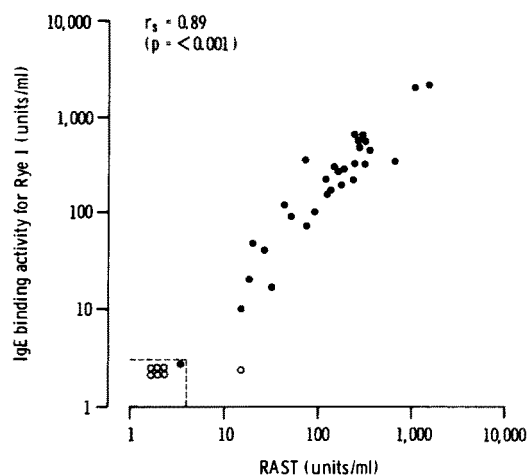


Figure 2. IgE ab measured by RAST with the use of Rye I rich pool (units/ml) and by antigen-binding radioimmunoassay with Rye I (IgE BA units/ml) in sera from patients with grass pollen hay fever (●). The two results were significantly correlated (Spearman rank correlation $r_s = 0.89$ $p < 0.001$). Sera from seven persons having negative skin tests for grass pollen extract (○) were also assayed by both techniques. The minimum level of IgE ab detectable by each assay is indicated (· · · ·).

To study the relationship between IgE BA and PK activity we tested the PK activity of these fractions in three nonallergic persons. To exclude interference by the recipient's own IgE (23, 24) we chose one recipient who was known to have no detectable serum IgE (<5 ng/ml). As shown in Table III we found that PK activity was similar in two recipients but was approximately 2-fold lower in the third. Calculating the concentration of IgE BA which gave a positive PK test showed that 2 to 4 units/ml were necessary for IgE from patient N.L. but that 1 to 2 units/ml were sufficient for IgE from the other two patients. The results (Table III) also confirm the absence of IgE ab in the IgG fractions. We also tested serum from two ragweed allergic patients who had received immunotherapy and whose serum was known to contain very high levels of IgE anti-

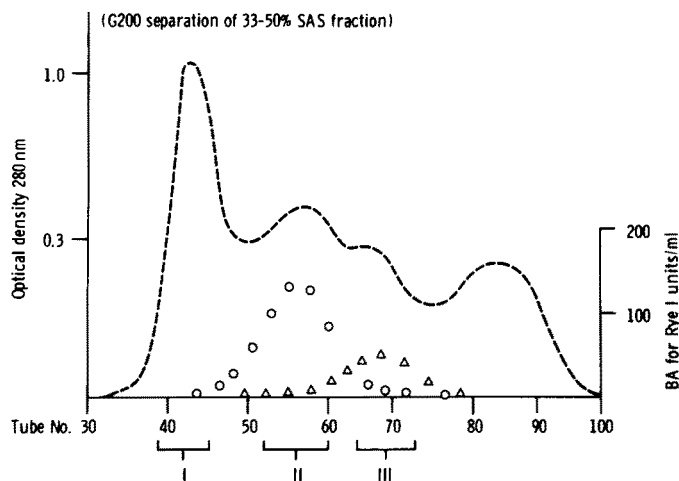


Figure 3. Sephadex G-200 separation of IgE ab to Rye I. The protein precipitated from 40 ml of serum N.L. between 33 and 50% SAS was dialyzed and passed through a 200-cm Sephadex G-200 column eluted with BBS, and collected in 6-ml fractions. Optical density was measured at 280 nm (-----). BA for Rye I was measured by radioimmunoassay precipitating with anti-IgG (Δ—Δ) or anti-IgE (○—○). Three fractions were pooled as shown.

TABLE II
Binding activity for Rye I^a in ammonium sulfate fractions^b of sera

	Whole Serum		SAS Fractions				Enrichment ^c
	IgG	IgE	0 to 33%		33 to 50%		
			IgG	IgE	IgG	IgE	
Untreated ^d							
A.D.	2900	2200	1860	270	324	1833	7.3
S.D.	287	40	220	14	36.5	24	4.7
T.B.	120	20	91	3.5	18	15	5.0
M.A.	1525	99	1250	20	155	78	7.5
N.L.	1375	912	1180	230	172	784	7.0
Treated ^e							
H.B.	32000	≈920	46000	600	11500	1750	≈6
D.W.	4800	<25	4700	<25	264	14	
R.H.	40000	<100	N.D. ^f	N.D.	2600	20	
Controls							
P.B.	187	<4	150	<4	25	<3	
S.M.	<25	<4	<25	<4	<3	<3	
T.M.	<25	<4	<25	<4	<3	<3	

^a Values are units/ml measured by radioimmunoassay using Rye I labeled with ¹²⁵I and antisera to IgG or IgE.

^b The protein ppt in 33% SAS and between 33 and 50% SAS was redissolved in BBS to 2½ times the original volume and where necessary further diluted before BA was assayed.

^c Enrichment of IgE BA relative to IgG BA in 33 to 50% fraction compared with original serum.

^d Patients with grass pollen hayfever who had never received immunotherapy.

^e Patients who had received immunotherapy.

^f N.D., not determined.

TABLE III
Binding activity for Rye I, total IgE, and PK activity in IgE-rich fractions

Patient	Fraction ^a	BA for Rye I			Total IgE ng/ml	PK Activity ^b Tested in		
		IgE	IgA	IgG		M.C. (36)	T.P.-M. (144)	G.R. (<5)
			units/ml					
N.L. untreated	Serum	912	125	1375	1190	N.T. ^c	200	N.T.
	Fraction II	800	62.5	25	960	400	200	400
	IgG	<6	<6	350	<6	N.T.	<5	N.T.
L.P.G. untreated	Serum	615	67	975	720	N.T.	N.T.	N.T.
	Fraction II	1237	110	<10	1680	1600	800	800
	IgG	<10	<10	1275	<6	<10	<10	N.T.
H.B. post-treatment	Serum	≈900	N.T.	32000	1010	800	400	N.T.
	Fraction II	1730	N.T.	2410	2050	1600	800	1600
	IgG	<200	N.T.	57000	<6	<10	<10	N.T.

^a Fraction II is the IgE-rich fraction from Sephadex G-200 separation of the 33 to 50% SAS fractions (Fig. 3). IgG is the protein precipitated by 33% SAS and not retarded by a DEAE-column equilibrated with 0.005 M phosphate buffer, pH 8.0.

^b PK titer is the reciprocal of the maximum dilution giving a ++ reaction when challenged 24 hr later. Values in parentheses are the total IgE ng/ml serum of the non allergic recipients.

^c NT, not tested.

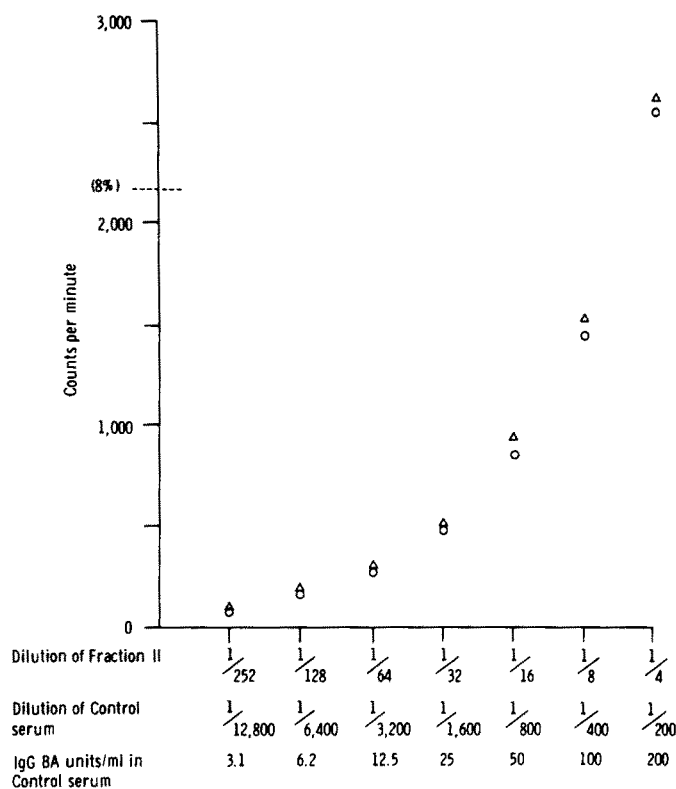


Figure 4. Comparison of IgG BA for Rye I in the control serum and IgE BA for Rye I in fraction II. Serial dilutions of control serum containing 40,000 units IgG BA/ml were assayed by using 3 ng ¹²⁵I Rye I and anti-IgG (○—○). Serial dilutions of fraction II were assayed by using ¹²⁵I-labeled Rye I and anti-IgE (Δ—Δ). The specific activity of the Rye I was 9200 cpm/ng at the time of this experiment, the value representing 8% binding of the added Rye I is shown by a dashed line.

Antigen E (measured by RAST elution (5)). Both sera had a PK titer of 1:16,000 when tested in a recipient whose serum IgE was 60 ng/ml and challenged with ragweed extract. One of these sera gave the same titer when tested in four other non-allergic recipients and challenged with antigen E. The IgG, IgA, and IgE BA in these sera were assayed as described previously (8) with modifications for IgE BA as shown in Figure 1. The

results in μg antigen E bound/ml were IgG BA 4.6 and 1.4; IgA BA $\approx$ 0.04 and 0.02 and IgE BA 0.32 and 0.33. Dividing the IgE BA by the PK titers suggests that IgE ab sufficient to bind 0.02 ng antigen E/ml can give a positive PK test.

Estimates of the quantity of IgE ab represented by 1 unit of IgE BA. Using three points on the section of the IgG BA control curve with less than 8% of the Rye I bound we calculated the Rye I bound/unit of BA. The quantity of IgG ab/unit of BA was then calculated by assuming that all the ab was in the state antigen₂ Ab (*Methods* and 17, 18). Under the conditions shown in Figure 1, the calculation suggested that 1 unit of IgG BA = 0.085 ng IgG ab and adjusting for the m.w. of IgE 1 unit of IgE BA = 0.1 ng IgE ab. As shown in Table I, when more Rye I was added to the assays increasing quantities of Rye I were bound per unit of BA when comparing tubes with marked antigen excess (<1.5% bound). Our experiments suggest that this increase reaches a plateau with about 0.3 μg /tube at which level antigen binding is 2.5- to 4.0-fold higher than with 3 ng/tube.

Previous estimates of the quantity of IgE ab present in sera have been made with modifications of RAST. This involves the use of antigen linked to a solid phase and clearly these conditions are not comparable with antigen binding in the fluid phase. For seven sera and fraction II of serum H.B. we compared RAST elution (5), RAST absorption (6), and the antigen-binding assay. The results in Table IV show that the RAST techniques correlate reasonably with antigen-binding results. Despite the small numbers of sera IgE BA was significantly correlated with the RAST elution results ($r_s = 0.93$ $p < 0.01$). As has been reported previously there was a good correlation between RAST and RAST elution (5) ($r_s = 0.97$ $p < 0.001$). In Table IV values are given for the percentage of IgE that is specific ab by assuming that 1 unit of IgE BA equals 0.1 ng of IgE ab. Applying this same conversion factor to the IgE BA results in Figure 2 and relating the results to the total serum IgE in those sera suggested that on average 8% of the IgE was specific ab to Rye I. It is clear, however, that the values in Table IV derived from IgE BA are lower than the values from RAST elution and RAST absorption. The RAST beads were made with Rye I-rich pool so that the higher values might have been partly due to the presence of antigens other than Rye I on the beads. However, taken together the results suggest that the

TABLE IV
Comparison between antigen binding, Rast elution and Rast absorption

Serum	Total IgE	IgE BA for Rye I ^a	RAST ^b	RAST Elution ^c	RAST Absorption ^d
	ng/ml	units/ml	units/ml	specific IgE ng/ml	% IgE absorbed
H.B. fraction II	2050	i ^e 1730 ii 1650 (8.3%)	1000	i 425 ii 605 iii 470 (24.5%)	i 55 ii 41
J.S.	8650	i 1034 ii 956 (1.1%)	960	i 625 ii 840 iii 725 (8.5%)	i 16 ii 21
D.C.	1215	340 (2.8%)	426	207 (17%)	33
V.W.	175	290 (17%)	184	65 (37.5%)	45
J.R.	1700	2160 (12.7%)	1560	815 (48%)	56
S.D.	1160	40 (0.4%)	26.5	8.1 (0.7%)	<5
A.D.	5500	2200 (4%)	1760	998 (18%)	11
M.E.	310	600 (19.5%)	370	96 (31%)	50

^a Values in parentheses are IgE ab calculated by using the conversion factor, (1 unit IgE BA = 0.1 ng IgE ab and is expressed as a percentage of total IgE).

^b RAST units obtained by reference to a standard curve with the use of HB fraction II which was defined as containing 1000 RAST units/ml.

^c The RAST elution technique was performed at pH 10.5 and calculated as described by Schellenberg and Adkinson (5). Values are specific IgE ab to rye grass pollen in nanograms per milliliter; results expressed as a percentage of the total IgE are given in parentheses.

^d RAST absorption carried out as described by Gleich and Jacob (6). Values are the quantity of IgE absorbed by Rye grass pollen immunoabsorbent minus the IgE absorbed by an HSA immunoabsorbent expressed as a percentage of the total IgE.

^e Results from repeat experiments are identified by: i, ii, iii.

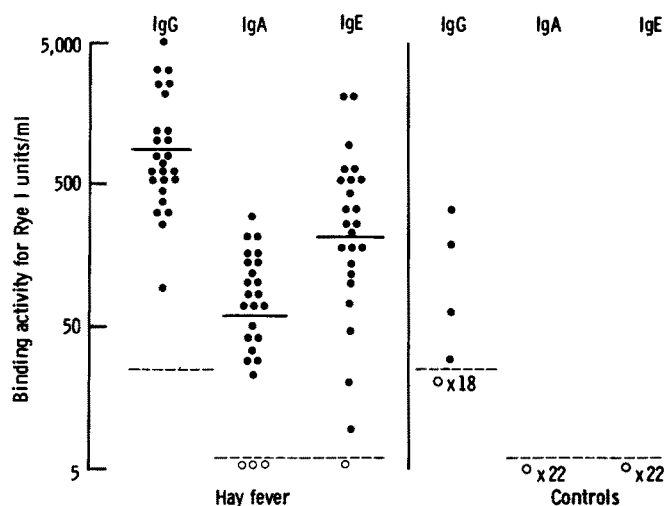


Figure 5. Antibodies to Rye I in the serum of patients with grass pollen hay fever and nonallergic persons. IgG, IgA, and IgE BA in units/ml was measured by antigen binding radioimmunoassay. Sera were obtained between October and December from patients who had never received immunotherapy.

antigen-binding assay is preferentially measuring high affinity ab and that RAST techniques are probably equivalent to an antigen concentration $>1 \mu\text{g/ml}$ in the fluid phase.

IgG, IgA, and IgE BA in the serum of patients with grass pollen hay fever. Binding activity for Rye I was assayed in the serum of 25 patients. The results show that IgG BA was

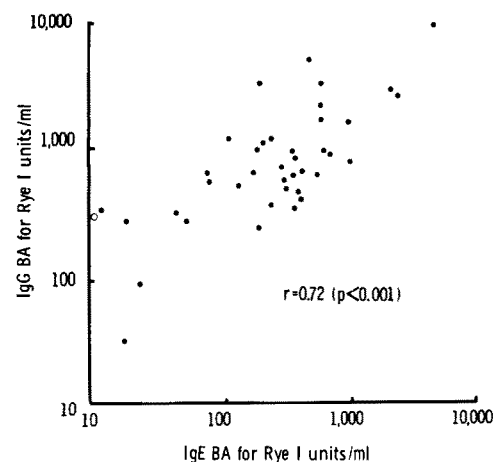


Figure 6. Correlation of IgE BA and IgG BA for Rye I in sera from patients with grass pollen hay fever who had never received immunotherapy. Correlation coefficient $r = 0.72$ ($p < 0.001$) ratio of geometric mean values 3.5:1.

detected in 25 of 25 sera, IgA BA in 21 of 25, and IgE BA in 24 of 25 (Fig. 5). The quantitative relationship between BA for the three classes was $\text{IgG} > \text{IgE} > \text{IgA}$ in 22 cases. Geometric mean values (G.M.) for IgG BA, IgE BA, and IgA BA were 869, 248, and 60.2 units/ml, respectively. The consistency of the relationship between the IgG and IgE BA suggested that there might be a direct correlation between them. Analyzing the results in Figure 5 plus results on sera from a further 15 patients who had

not received immunotherapy showed a highly significant correlation between IgG BA and IgE BA ($r = 0.72$ $p < 0.001$, ratio of G.M. 3.5:1) (Fig. 6). IgM BA for Rye I was assayed in 10 sera; although IgM BA was detected in five sera this was low— <20 units/ml and certainly IgM BA was lower than any of the other classes. None of the 22 negative control sera assayed contained detectable IgE or IgA BA, but four sera contained IgG BA and in two cases this was within the range seen in serum from patients with hay fever (see Fig. 5). In a further series of 26 persons without hay fever we have seen six sera with detectable IgG BA but in each case IgG BA was less than 1000 units.

Fifteen patients were treated with 9 to 11 graded injections of alum-precipitated pyridine extract of grass pollen (13). After this treatment their sera were assayed in parallel with pretreatment sera. Where necessary IgE and IgA BA were assayed in 33 to 50% SAS fraction, the values being adjusted according to the proportion of serum IgE and IgA in the fractions. The results left no doubt that the increased levels of IgG BA (G.M. 966 units pre- and 8705 units post-treatment) were not matched by changes in IgE BA (G.M. 216 units pre- and 286 units post-treatment) or IgA BA (G.M. 53.4 units pre- and 129 units post-treatment). On average the ratio of IgG BA to IgE BA increased from 4.1:1 before treatment to 30:1 after treatment.

DISCUSSION

In 1958 Skom and Talmadge reported an antiglobulin modification of the Farr Technique for measuring non-precipitating antibodies (19, 25). Subsequently Mulligan *et al.* showed that antigen-binding capacity could be used to make estimates of the quantity of ab in a serum (18) and Osler *et al.* (26) demonstrated antigen-binding activity in IgM- and IgG-rich fractions of serum. Newcombe and Ishizaka showed that selective precipitation of IgG or IgA from serum would allow estimates of class-specific antigen-binding capacity (15). When IgE was discovered it was shown that IgE ab could bind pollen antigens but because of the small amounts of total IgE the precipitate had to be stabilized with agar (1). Zeiss *et al.* overcame the problem of low levels of IgE by allowing radiolabeled antigen to react with IgE anti-IgE complexes on the surface of assay tubes (3). However, these techniques could not be directly compared with assays for other classes of ab (1, 3). RAST can give quantitative values for IgE ab (5, 6). Although RAST assays have been reported for human IgG and IgA ab, these assays are not comparable to the IgE ab RAST (27–29). We have confirmed (data not shown) the experience of other laboratories that background binding of IgG and IgA to RAST beads is too high to allow sensitive assays (30, 31). The sensitivity of antigen-binding assays was inversely related to the quantity of antigen added (Table I, and 3, 15, 19) and had increased because the specific activity of radiolabeled antigens had increased (12). Previous assays for IgG ab have used larger quantities of antigen than those used here, e.g., 100 μ g HSA (18); 2 μ g diphtheria toxoid (15), and 1 μ g antigen E (32). It has been reported that both IgE ab and γ -globulin will bind less antigen with antigen concentrations below 1 μ g/ml (3, 19). In the present assay we have used 3 ng of antigen/tube (10 ng/ml) because this concentration was found to give maximum sensitivity. In keeping with the previous results (3, 19) we found that all three classes of ab would bind more antigen when the concentration present was increased (Table I). This increase was not due to an increase in the degree of antigen excess since it was seen comparing tubes with a very large excess of antigen (Table I). The validity of the assay described here depends on the fact that the increase in antigen binding with increasing

antigen concentration is very similar for different classes of ab within a given serum and also for different sera. The implied conclusion that these sera from untreated patients have similar avidity is supported by the good correlation between IgE BA and RAST results (Fig. 2) (33).

We have estimated that 8% of the total IgE in English patients with grass pollen hay fever is IgE ab to Rye I. Applying RAST elution values (see Table IV) or using antigen-binding values obtained with 0.1 μ g Rye I/assay would give an average value of $\approx 25\%$. Clearly our RAST results may be high because we used Rye I-rich pool instead of Rye I. However, the RAST results appear to be broadly equivalent to antigen-binding results in the presence of 1 μ g Rye I/ml. The positive control serum used here had previously been compared with a rabbit antiserum to Rye I which had been standardized by quantitative precipitation with Rye I at equivalence (34). That estimate suggested that 1 unit of IgG BA ≈ 0.7 ng IgG ab which is similar to the highest values obtained by RAST absorption (Table IV). It is unlikely that concentrations of pollen antigens as high as 1 μ g/ml ever occur due to natural exposure. Certainly both intradermal skin tests and *in vitro* histamine release from leukocytes can often be achieved with $<10^{-5}$ μ g/ml of pollen antigens (34, 35). We feel that the assay described here measures a consistent and presumably high affinity fraction of the antibody response to grass pollen. The relationship between different sera might vary slightly if higher antigen concentrations were used but the results accurately reflect quantitative differences between classes. Our estimates of the quantity of IgE antibody confirm previous results that a high proportion of total IgE may be directed against pollen antigens (see Table IV) (1, 3, 5, 6). As shown by Gleich and Jacob, increases in IgE ab to pollen antigens may well be sufficient to cause increases in total IgE (6).

Considering that PK activity is tested in 2-fold dilutions, the variations found here in correlating IgE BA with PK activity were not very great. A variety of factors affecting histamine release might explain the reduced sensitivity of one recipient (34); alternatively this might have been related to the higher serum IgE of this recipient as shown by Bazaraal *et al.* (23). We also found that by using IgE from one patient (N.L.) approximately 2-fold more IgE BA was required to elicit a PK test. This result was not expected since the source of IgE has not been found to affect the affinity of IgE for basophils (36). The data shown in Table III show that the variation observed here is unlikely to be due to nonspecific IgE (24) or to the effect of other classes of antibody. The minimum concentration of IgE BA that gave a positive PK test in these experiments was 1 unit/ml, which we estimate represents 0.1 to 0.3 ng IgE ab/ml. This estimate is close to the value obtained by applying a similar calculation (see *Methods*) to the previous data (1). The results of RAST absorption on Fraction II of serum H.B. suggest that as much as 0.6 ng IgE ab/ml might be required for a positive PK test (Tables II and IV). For the sera from patients with ragweed hay fever we calculate that a dilution of serum containing IgE ab sufficient to bind 0.02 ng antigen E/ml will give a positive PK test. Under similar conditions 1 unit IgE BA/ml will bind approximately 0.025 ng Rye I/ml. Thus, the minimum estimate for the Rye I system is in good agreement with the results for the antigen E system. Our results show that PK testing can be more sensitive than either RAST or the antigen-binding assay.

The antigen-binding assay described here is not difficult to carry out and in principle is applicable to any antigen that is available in sufficiently pure form and can be radiolabeled. The major disadvantage of the assay is that it requires large quan-

titities of specific antisera and IgE myeloma serum. The assay would appear to be particularly suitable for comparing IgE, IgA, and IgG ab in serum or secretions of untreated patients with the levels in nonallergic persons. Previous quantitative reports of IgG and IgE ab have used different techniques for the two classes (32, 37). Thus, Yunginger and Gleich (32) compared RAST results with IgG antigen-binding capacity; they reported that immunotherapy caused a selective rise in IgG ab. However, their IgG ab assay was less sensitive than that reported here and they did not find a clear distinction between IgG ab in untreated allergic persons and nonallergic persons (32). The absence of detectable ab of any class in the serum of most nonallergic persons (Fig. 5) confirms previous results for IgG and IgA ab with radioimmunoelectrophoresis (1, 2, 38) and our own data on nasal washings (8). For both antigen E and Rye I most nonallergic sera show no significant BA above a background level defined by myeloma sera. Sera from English patients who had not been exposed to ragweed and sera from the majority of nonallergic persons in Baltimore gave very similar background levels for IgE BA, IgA BA, and IgG BA for antigen E (8, 34). Our results confirm previous results that a proportion of nonallergic persons (10/48 = 20%) have IgG ab to pollen antigens without having IgE ab (34, 39). We feel that some previous results suggesting the presence of IgG or IgA ab in the majority of nonallergic persons have been due to underestimates of the variation in normal globulin background of the assays used (28-30, 40). Using the assay reported here we have confirmed that the nonallergic persons without serum antibodies to Rye I also have no detectable IgE, IgG, or IgA BA in their nasal secretions (T.A.E. Platts-Mills, The relationship between IgG, IgA, and IgE antibodies in serum and secretions. Clin. Allergy 8:95, 1978). The high incidence of IgG, IgA, and IgE BA in serum of patients with hay fever is in keeping with previous results (1, 2, 38) and confirms the importance of Rye I as an allergen in England (11, 41). The high levels of IgE BA relative to IgG BA were remarkable; in several sera IgE BA represented 40% of the total BA. This is clearly very atypical since total IgE is usually only 1/10,000 of serum IgG. The consistent relationship between BA in different classes, IgG>IgE>IgA>IgM, was also very striking. It has previously been reported that IgG ab after immunotherapy is directly correlated with IgE ab (32). We have found a significant correlation between IgG BA and IgE BA in serum from untreated patients ($r = 0.72$ $p < 0.001$) (Fig. 6). In keeping with this finding we have never seen IgE or IgA BA in the absence of IgG BA. Thus, despite the relatively high levels of IgE BA in the immune response to pollen antigens, the response appears to include all three classes. These findings support the view that the genetic or other differences between allergic and nonallergic persons affect antigen recognition or antibody production in general rather than being differences concerning IgE production alone.

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