

Measurement of IgG antibodies to house dust mite and grass pollen by a solid-phase radioimmunoassay

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Summary

Polystyrene microtubes, coated with extracts of either *Dermatophagoides pteronyssinus* (DPT) or grass pollens, were used as the solid-phase in a radioimmunoassay for the measurement of specific IgG antibodies to the corresponding allergen. Radiolabelled protein A from *Staphylococcus aureus* (SpA) was used to determine the IgG antibodies attached to the tubes. The binding of IgG from either normal or allergic sera to DPT-coated tubes was antigen specific and mediated by the Fab fragment of the immunoglobulin. IgG antibodies purified by affinity chromatography from non-allergic serum competed with IgE antibodies to DPT.

IgE antibodies do not significantly interfere with the assay. Indeed, heating a reaginic serum resulted in a striking reduction of the (^{125} I) anti-IgE binding to allergen-coated tubes without modifying the (^{125} I)-SpA binding. Furthermore, filtration of a reaginic serum through Sephacryl S-200 separated a peak of IgE antibodies, characterized by a high binding of labelled a-IgE and a low binding of (^{125} I)-SpA from the peak of IgG antibodies defined by low a-IgE and high SpA binding.

The solid phase method is more sensitive than a double-antibody technique employing the same DPT extract as labelled antigen. Non-allergic subjects had less IgG antibodies to DPT or grass pollens than allergic patients.

In untreated patients, there was a good correlation between levels of IgG and IgE antibodies to grass pollens but not to DPT. Patients hyposensitized to house dust mite had on the average three times more specific IgG antibodies than untreated cases.

Introduction

Mites of the *Dermatophagoides* species constitute the major allergen of house dust (Voorhorst, Spijksma-Boezema & Spijksma, 1964; Voorhorst *et al.*, 1967; Maunsell, Wraith & Cunningham, 1968; Holford-Strevens *et al.*, 1970).

Crude extracts of *D. pteronyssinus* (DPT) or of *D. Farinae* are currently used for

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both *in vivo* and *in vitro* diagnosis and treatment of allergic patients. Attempts to purify mite allergens have shown that an allergenic activity was present in several constituents of the crude extract differing either by their molecular weight (Miyamoto, Oshima & Ishizaka, 1969; Romagnani *et al.*, 1972, 1976; Holford-Strevens, 1972), their ionic charge (Romagnani *et al.*, 1976; Ricci, Biliotti & Romagnani, 1975) or their affinity for lectins (Baldo & Uhlenbruck, 1977). The most allergenic preparations were obtained by gel filtration of the crude extract and concentration of the fractions with a molecular weight between 15–20,000 daltons. Very recently, Chapman & Platts-Mills (1978) using a combination of Sephadex G75 filtration and Pevikon electrophoresis succeeded in preparing a more purified fraction with a skin reactivity comparable to that of the crude extract. This fraction could be radiolabelled and used as antigen in a sensitive double antibody radioimmunoassay allowing the determination of antibodies to DPT of the IgG, IgA and IgE classes.

In the present report we describe a simple solid-phase radioimmunoassay for measuring IgG antibodies to DPT and grass pollens. The main advantage of this procedure is that it does not necessitate highly purified allergen extracts. We also show that specific IgG antibodies to DPT are found in the serum of healthy non-allergic subjects.

Materials and methods

Reagents

Allergen extracts were kindly provided by Dr D. A. Hordle (Beecham Research Laboratories, England). Two batches of *D. pteronyssinus* were used, they were comprised of a crude extract, extensively dialysed against PBS and distilled water, and of a more purified fraction (R 537), obtained by filtration through Sephadex G75. A mixture of equal amounts of freeze dried extracts of the following grass pollens was used throughout the study: fescue, meadow, rye grass, dogstail and foxstail.

Crude extract of either DPT or pollen was linked to sepharose 2B by the cyanogen bromide technique (Axen, Porath & Ernback, 1967). Human serum albumin (HSA), ovalbumin, bovine serum albumin, twice crystallized, were purchased from Sigma; alpha lactalbumin was obtained from Nestlé Lab. (Switzerland). Human IgG has been prepared from a pool of forty blood donors by ammonium sulphate precipitation followed by DEAE cellulose chromatography. The first peak obtained by elution with phosphate buffer 0.005 M pH 8 was concentrated and digested with pepsin as described by Campbell *et al.* (1970). F(ab')₂ fragments were purified by Sephadex G100 chromatography. Protein A, from *Staphylococcus aureus* and radiolabelled goat anti-human ϵ_2 antibodies were obtained from Pharmacia (Uppsala, Sweden).

Coating of the tubes with allergens

Polystyrene tubes (Removawell M74A, Cooke Lab.) could be fitted into the support of a standard microplate. For coating with antigen, the tubes were filled with 0.2 ml PBS containing various concentrations of allergen or 50 μ g/ml HSA. After an overnight incubation at 4°C, the tubes were washed and filled for 2–4 hr with 0.2% gelatin in PBS (pH 7.2). This was followed by another wash prior to use in the assay. It is important to note that 0.5 l PBS was used to wash the 96 tubes of a plate. Washes were performed with a Skatron cell-harvester.

Handling of the sera

Sera containing NaN_3H could be stored for 1 week at 4°C , afterwards they were frozen at -40°C . Reference sera obtained from young hyposensitized patients were divided in aliquots and frozen at -40°C ; pooled sera from non allergic blood donors were absorbed either on sepharose-DPT or on sepharose-pollen immunosorbent and then similarly stored. All the sera were centrifuged 30 min at 3 000 g prior to use.

Labelling of protein A

Protein A (125 μg) in 50 μl PBS was iodinated with 5 mCi (^{125}I)Na by the chloramine T method described by Klinman & Taylor (1969). Unbound ^{125}I was eliminated by dialysis. Labelled protein A was stored at 4°C in PBS containing 0.02% tween and 0.2% gelatin. 4 ng of (^{125}I) protein A yielded about 100,000 ct/min. Before use, protein A was centrifuged at 3000 g for 30 min. 20 μg of purified DPT (R537) were labelled by using exactly the same procedure.

Radioimmunoassay

Each measurement was done in duplicate. Two hundred μl of serum at the appropriate dilution in PBS $\bar{c}$ 0.02% tween was incubated in allergen and HSA-coated tubes for 18 hr at 4°C in a moist chamber. The tubes were then washed with the Skatron cell-harvester and were again incubated overnight with 200 μl (^{125}I) protein A ($\pm$ 100,000 ct/min., in PBS tween). After a further wash they were counted in a gamma counter. For each assay a background was obtained by using PBS instead of serum in the first incubation step; this blank was always less than 4% of the total radioactivity.

IgE antibodies

IgE antibodies were measured by the classical RAST technique employing the Phadebas kits or by counting the amount the radiolabelled anti- ϵ_2 antibody fixed to the antigen coated tubes. Polyclonal IgE were measured by the PRIST technique employing the Phadebas reagents.

Double antibody radioimmunoassay.

Fifty μl of the reference serum, diluted 1/40 in PBS, were incubated for 3 hr with 50 μl (^{125}I)-DPT (70,000 ct/min). Fifty μl of goat antihuman IgG was then added and the mixture was incubated overnight at room temperature. The precipitate was washed and counted as previously described (Delespesse *et al.*, 1976). The blank consisted in normal human serum absorbed on sepharose-DPT and used at the dilution of 1/40. Successive two fold dilutions of the reference serum were made in PBS $\bar{c}$ 2.5% absorbed normal human serum.

Results*Evidence for an antigen specific IgG binding to DPT coated tubes in sera from allergic and non allergic subjects*

In a first series of experiments the sera from twelve blood donors have been compared to those of nine asthmatic patients hyposensitized with house dust mite extracts (Bencard, SVD-R). The blood donors had negative skin and RAST tests against ten common allergens (including DPT and *D. farinae*); by contrast all the patients displayed at least a score two against DPT in each test.

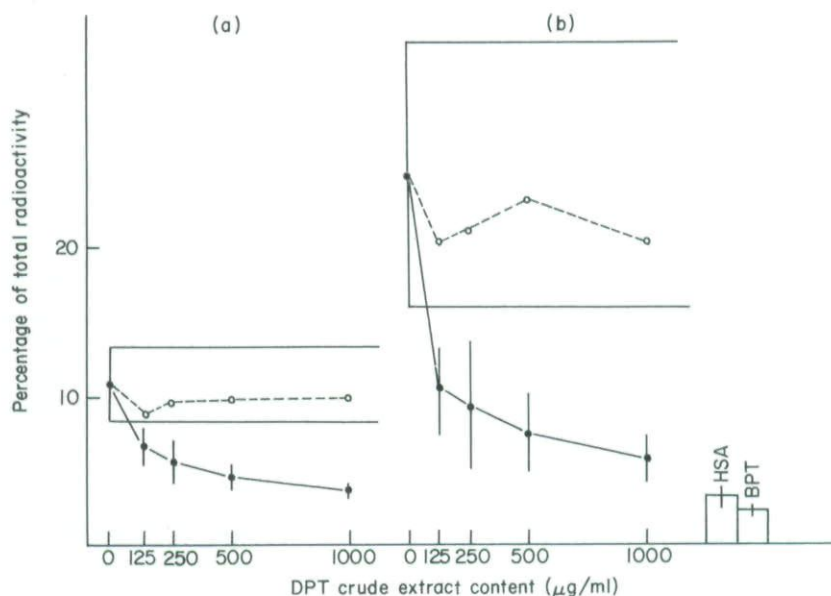


Fig. 1. Binding of IgG from normal (a) and allergic (b) subjects to DPT coated tubes. Results are expressed as a percentage of the total radioactivity $\pm$ s.d. The sera diluted 1/10 were mixed with one volume of PBS containing 0 to 1·000 μ g/ml DPT crude extract (●—●) or alpha lactalbumin (○—○). The mean binding $\pm$ s.d. of the twenty-one sera to HSA-coated tubes and the blank are represented by the open column.

The sera diluted 1/10 were mixed with one volume PBS tween containing various amounts of either DPT crude extract or alpha lactalbumin; controls were diluted 1/20 in PBS tween. After an overnight incubation at 4°C these mixtures were processed as described in 'Materials and methods'. Two conclusions can be drawn from this experiment (Fig. 1):

The mean value of the binding to DPT-coated tubes was significantly higher in the sera from allergic than from non-allergic subjects (mean ct/min $\pm$ s.e. mean were respectively $24\cdot807 \pm 4\cdot727$ and $10\cdot800 \pm 1\cdot162$). By contrast the binding to HSA-coated tubes was identical in the two groups of sera ($2\cdot740 \pm 917$ and $2\cdot581 \pm 1\cdot199$).

A clear dose-dependent inhibition was observed in the sera preincubated with DPT crude extract while no significant modification of the IgG binding was noted in the same sera treated with alpha lactalbumin.

In order to assess further the specificity of the IgG binding, comparable inhibition assays were performed with five different antigens on two sera from each group of subjects and on three sera from patients with IgG myeloma. The latter had been pre-diluted in order to contain 10 mg/ml IgG, which was estimated to represent the normal IgG level.

As can be seen in Fig. 2, a significant inhibition was obtained with 5 μ g/ml purified DPT extract while no effect was noted with 500 μ g/ml bovine serum albumin, ovalbumin, PPD or streptokinase-streptodornase. Note that no inhibition was obtained in the myeloma sera which displayed a similar binding on DPT- and HSA-coated tubes.

These experiments suggest the existence, in normal and allergic sera of IgG

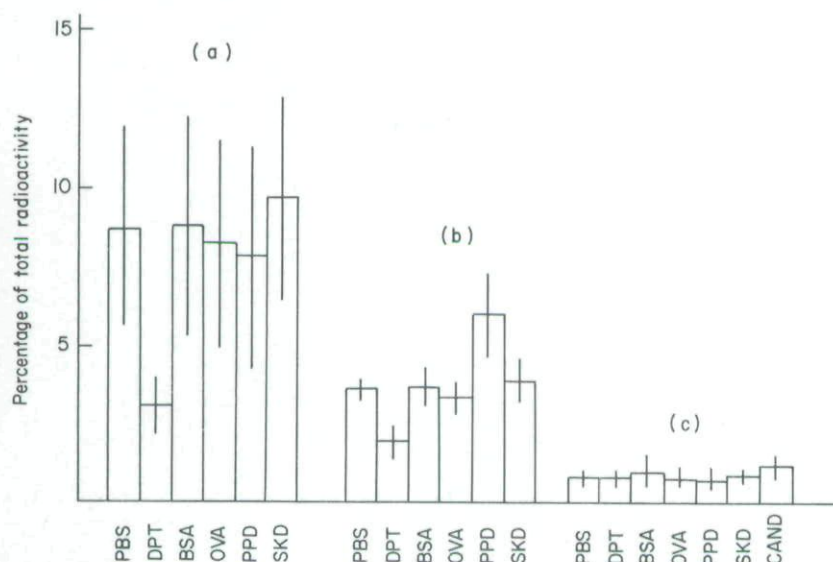


Fig. 2. Specificity of the IgG binding to DPT coated tubes. Results are expressed as a percentage of the total radioactivity $\pm$ s.d.; (a), sera from allergic patients; (b) sera from normal subjects; (c), IgG myeloma. The first column corresponds to sera diluted 1/40 in PBS, the other columns represent sera diluted in PBS containing 5 μ g/ml DPT (R 537), or 500 μ g/ml of the following: bovine serum albumin (BSA), ovalbumin (OVA), purified tuberculin (PPD), streptokinase-streptodornase (SKD), candidine (Cand.).

molecules capable of binding specifically to DPT extract coating the polystyrene tubes.

This conclusion is further supported by the observation that the IgG binding of normal or allergic sera was related to the DPT concentration of the solution used to coat the tubes (Fig. 3). Such a dose-response curve was not noted when myeloma sera were tested.

Evidence showing that IgG binding to DPT coated tubes is mediated by Fab fragment

If normal sera really contain specific IgG antibodies against DPT (IgG-a-DPT), then F(ab')₂ fragments of IgG purified from a pool of non-allergic blood donors should compete in the solid phase assay with IgG antibodies present in the serum of hypo-sensitized patients. This has been shown by preincubating the DPT-coated tubes with 0.2 ml PBS containing 500 μ g F(ab')₂; control tubes were similarly incubated with PBS. After an overnight incubation at 4°C the tubes were washed and used to test six allergic sera as described in 'Materials and methods'.

The results (Table 1) clearly showed an important reduction of the IgG binding to DPT in tubes preincubated with F(ab')₂. In another experiment we further observed that DPT IgG antibodies purified by affinity chromatography competed with IgE antibodies present in the serum of an untreated allergic patient (C). Forty ml serum from a blood donor with a negative RAST and skin test to DPT, were passed through a column containing 30 g sepharose-DPT. After washing with 600 ml PBS, the column was eluted with glycine buffer pH 2.8. The eluate (E) was neutralized by TRIS 1 M pH 9, dialysed against PBS and finally concentrated under vacuum. One volume of serum C, diluted 1/10, was mixed with one volume of either PBS or PBS containing two fold

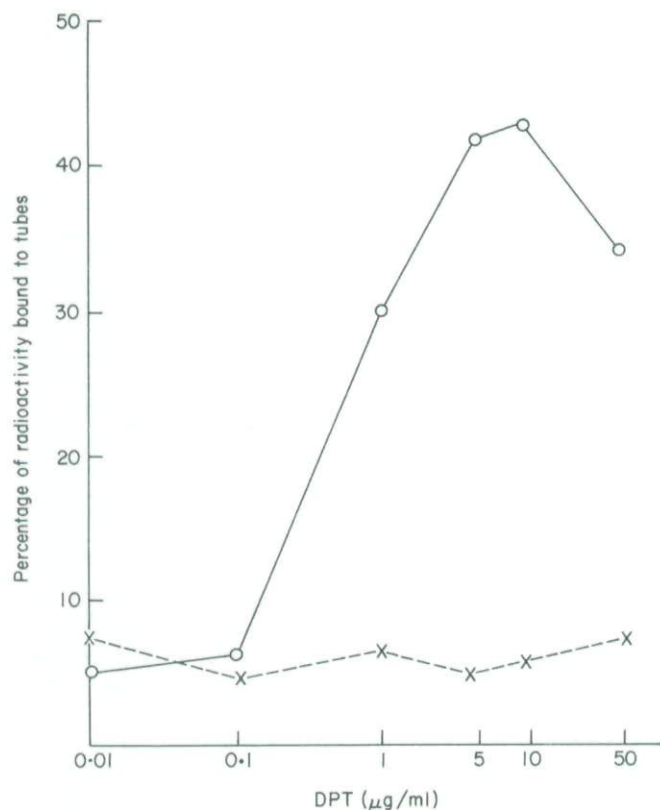


Fig. 3. Relation between the amount of DPT used to coat polystyrene tubes and the IgG binding. The concentration of R 537 used to coat the tubes is indicated on the abscissa, the ordinate represents the percentage of radioactivity bound to the tubes. Results from an allergic serum (○—○) and IgG myeloma (x---x) are presented.

Table 1. Inhibition of IgG antibody binding to *D. pteronyssinus* by F(ab')₂ fragments of normal IgG

Exp.	IgG binding to <i>D. pteronyssinus</i>		
	In the absence of F(ab') ₂	In the presence of F(ab') ₂	Inhibition (%)
1	40.967	20.075	50
2	27.271	16.997	38
3	17.806	13.393	25
4	38.756	18.994	51
5	31.981	15.246	53
6	22.886	9.149	61

The tubes coated with purified *D. pteronyssinus* were filled with 0.2 ml PBS or 0.2 ml PBS containing 0.5 mg F(ab')₂. After an overnight incubation at 4°C and washing, the tubes were used as described in 'Materials and methods'. Results are expressed in c/min.

Table 2. Competition between IgG and IgE antibodies to *D. pteronyssinus*

	Bound ^{125}I a-IgE*	Inhibition (%)
Serum C + PBS	13.953 $\pm$ 439†	—
Serum C + E	9.654 $\pm$ 522	31
+ E 1/2	11.267 $\pm$ 381	22
+ E 1/4	14.122 $\pm$ 594	0
E	120 $\pm$ 31	—

* The total radioactivity added to each tube was 117,249.

† net ct/min $\pm$ s.d.; blank (981 $\pm$ 274).

dilutions of E, then 0.2 ml of these mixtures were introduced into DPT coated tubes. After an overnight incubation the tubes were washed and filled with 0.2 ml of (^{125}I) anti-IgE; 18 hr later they were washed and counted. The results (Table 2) indicated that mixtures containing E yielded a lower binding of (^{125}I) anti-IgE. It can be concluded from the above data that IgG antibodies detected by the solid-phase assay bind to DPT through their F ab fragment and that they recognize, at least in part, the same antigenic determinants as the IgE antibodies to DPT.

Interference of IgE antibodies with the solid phase radioassay

Following the recent demonstration by Johansson & Ingamas (1978) that SpA binds to polyclonal IgE, we showed that this phenomenon was not interfering with our assay. As indicated in Table 3, heating the serum of a patient allergic to DPT, at 56°C for 30 min resulted in a significant reduction of the labelled anti- ϵ_2 binding but had no effect on the (^{125}I)-SpA binding. These results indicate that SpA does not bind to the heat labile region of IgE molecules but do not exclude the possibility of its binding to F ab region of these antibodies.

In the experiment reproduced in Fig. 4 a reaginic serum was filtered through a Sephacryl S-200 column and the different fractions were assessed for their content in both polyclonal and pollen specific IgE and IgG antibodies. It is evident that the peak

Table 3. Influence of heating the serum on the measurement of IgE and IgG antibodies against *D. pteronyssinus*

	IgE a	IgG a
Total radioactivity	113.435*	82.276
Blank (ct/min)	353	573
Serum (ct/min)	23.975	26.852
Heated serum (ct/min)	8.919	30.981

* = ct/min.

† = 56°C, 30 min.

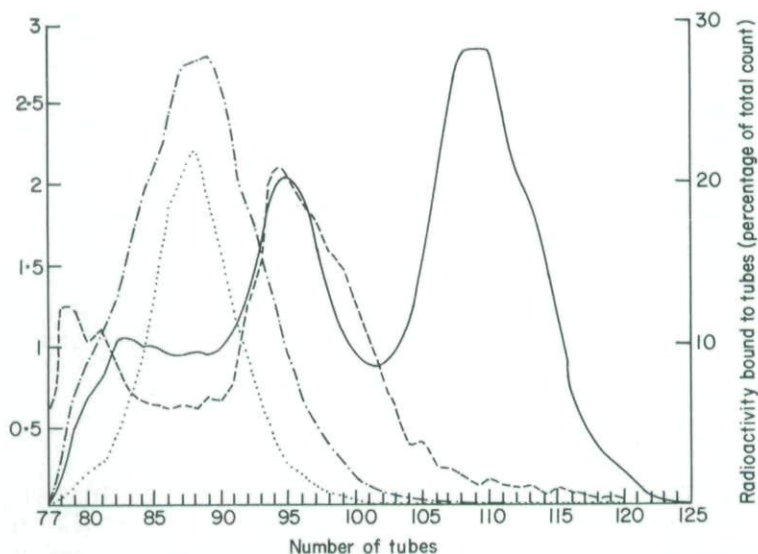


Fig. 4. Two ml serum, rich in IgE-a-pollen, was separated on a 3.2×110 cm Sephacryl 200 column (6 drops/min, 40 drops/tube). Protein was determined by u.v. absorption at 280 nm (—) (left ordinate). The IgE concentration of every second fraction was measured by PRIST test (— · — · —), IgE-a-pollen (· · ·) and IgG-a-pollen (— · — · —) were determined by the plastic tube assay as described in 'Materials and methods'. Results are expressed in terms of radioactivity bound to the tubes (right ordinate; percentage of total count). Polyclonal IgG peak in the 94th tube. IgG was determined by Mancini analysis (not shown).

of IgE antibodies detected by $(^{125}\text{I})\text{a-IgE}$ is distinct from that of IgG antibodies evidenced by $(^{125}\text{I})\text{-SpA}$. Worth noting is the existence of a first peak of antibodies detected by $(^{125}\text{I})\text{-SpA}$ and eluted in the void volume. This might correspond to IgM, Ig aggregates or immune complexes. The first possibility seems to be the most likely as we could not find any C1q binding activity in this peak (not shown). Data from Harboe & Fölling (1974) further support this explanation by showing the affinity of SpA for human IgM and IgA.

Standardization of the solid phase assay

A standard curve was constructed by serial twofold dilutions of the reference serum. The dilutions were made in PBS tween containing 1% normal serum absorbed on sepharose-DPT. This diluent was used to calculate the blank which was always less than 3% of the total radioactivity. The initial dilution was 1/40 PBS tween. As shown in Fig. 5, a final dilution of 1/5120 was still significantly different from the background. Multiple trials have been performed to minimize the non-specific binding of IgG to the plastic tubes. We have systematically investigated different conditions for storing the serum, its optimal dilution, the pH, the ionic strength and the protein content of the solutions used to wash or coat the tubes. The optimal conditions are those described in 'Materials and methods'; the following methodological points seemed particularly important:

- (i) The serum and the labelled protein A must be centrifuged (3000 g, 30 min) immediately before use, in order to eliminate the large aggregates.

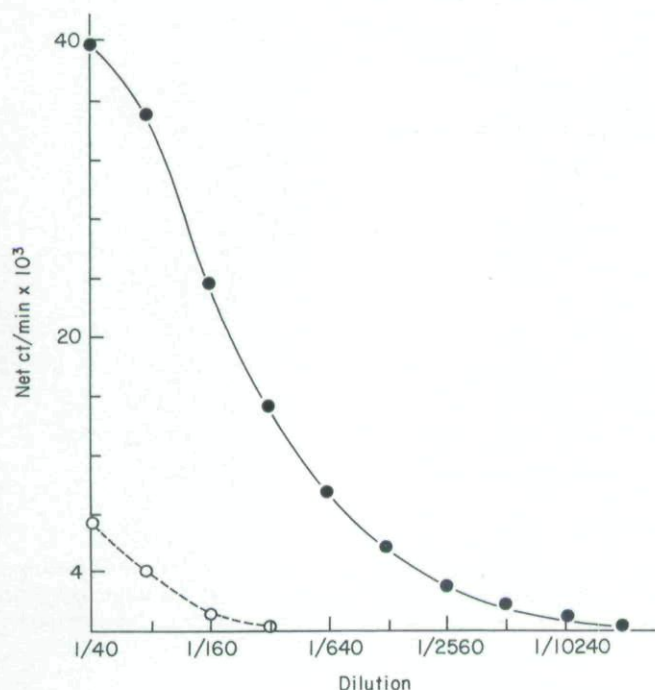


Fig. 5. Standard curve for IgG antibody measurement. The reference serum is diluted in PBS containing 0.02% tween 20 and 1% normal serum (absorbed to DPT-sepharose). Initial dilution is 1/40. Net ct/min are indicated on the ordinate; the abscissa represents the inverse of the dilution of the reference serum. Lower curve (o . . o) represents ct/min precipitated in the double antibody method.

- (ii) The washes must be abundant; 0.5 litre PBS was used to wash ninety-six microtubes fitted into one microplate.
- (iii) The sera may be stored at 4°C for one week; afterwards they must be frozen at -40°C.
- (iv) The individual background of each serum, determined by its binding on HSA-coated tubes, must not be higher than that of the absorbed normal serum.
- (v) For routine assays, the sera were diluted 1/100 for determination of antibodies to DPT and 1/40 for pollen antibodies.

By respecting these technical conditions we have estimated the reproducibility of the assay on twelve sera tested four times at weekly intervals and found a mean coefficient of variation of $19.1 \pm 4.9\%$.

Comparison of the solid phase assay with a double antibody method

Standard curves obtained by each procedure are shown in Fig. 5. The last dilution of the reference serum significantly different from the background was 1/160 in the double antibody method and 1/5120 in the tube assay. Similar results were reproduced by testing serial dilutions of other sera from hyposensitized patients.

Comparison of IgG and IgE antibody levels

Sera from normal donors and from allergic patients with no history of previous

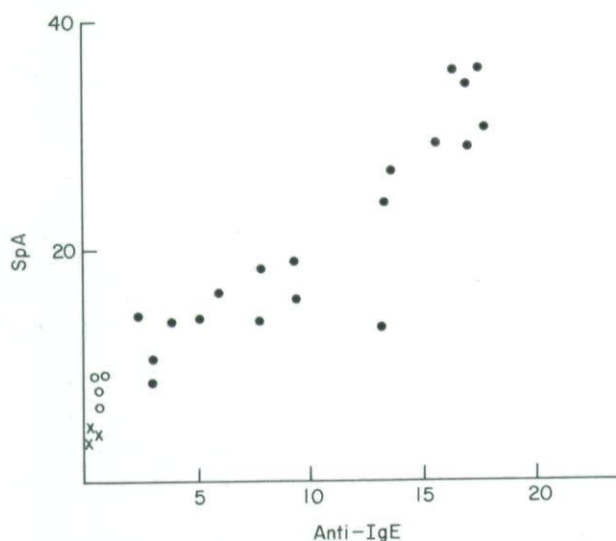


Fig. 6. Correlation between IgE- and IgG-a-pollen in sera from patients who had never received immunotherapy (●), in normal sera (○) and IgG myeloma sera (×). The binding of labelled anti-IgE and SpA, expressed as a percentage of the total radioactivity, are indicated respectively on the abscissa and on the ordinate. Total radioactivity was 102,000 ct/min for (^{125} I)-SpA and 94,000 for (^{125} I)a-IgE.

Table 4. IgG-a-DPT in sera of various origins

Sera	IgG-a-DPT*
Non allergic (19)†	113 ± 232
Allergic untreated (17)	257 ± 171
Allergic desensitized (21)	642 ± 374

* Expressed in arbitrary units/ml

† Number of cases.

hyposensitization against grass pollen, have been tested for their content in IgE and IgG antibodies directed against the mixed grass pollen preparation. As shown in Fig. 6 a good correlation was observed between these two classes of antibody to grass pollen. Conversely, no correlation could be found between levels of IgE and IgG antibodies to DPT (not shown). However it must be noted that patients allergic to house dust mite, as demonstrated by a RAST score ≥ 2 , displayed higher IgG-a-DPT than non-allergic controls (Table 4). Furthermore among the group of allergic subjects, those hypsensitized for at least one month had on the average three times more IgG-a-DPT than untreated ones.

Discussion

The above data clearly show that the polystyrene coated tube assay may be used to detect specific IgG antibodies to DPT. Indeed we have described experimental conditions where more than 95% of IgG binding to allergen-coated tubes is antigen specific

and mediated by the F ab fragment of IgG. It is known that the major problem limiting the use of solid-phase assay for the measurement of IgG or IgA antibodies is the high non-specific binding of these immunoglobulins to the solid-phase.

We have overcome this difficulty by using polystyrene tubes and radiolabelled SpA in place of RAST beads or RAST paper discs and labelled anti-IgG. The two main advantages of the present method are: (i) crude extracts (pollens) or semi-purified preparations (DPT) of allergens may be used. We have shown that the DPT extract, employed to coat the tubes, was weakly precipitated in the double antibody method. The most likely explanation is that the labelled allergen was not pure enough for this antigen-binding assay, which, as a consequence, was twenty to thirty times less sensitive than the tube technique; (ii) the use of small tubes fitting in a microplate permitting both an economy of the reagents and a rapid and abundant wash with a cell-harvester. Although it is generally accepted that SpA specifically binds to IgG, it has been shown to interact only with the IgG subclasses 1, 2 and 4. (Forsgren & Sjoquist, 1969). Thus IgG₃ antibodies cannot be detected by the above assay. Furthermore, the IgG specificity of SpA has been recently questioned (Harboe & Fölling, 1974) so that it could be that IgM or IgA antibodies are also detected in our method.

The present results indicate that IgE antibodies do not significantly influence the measurement of IgG antibodies. These findings are important in regard to the recent demonstration by Johanson & Ingamas (1978) that SpA interacts with 6 to 8% of polyclonal human IgE.

The systematic use of HSA-coated tubes in parallel with allergen-coated tubes indicates that in the great majority of sera tested up to now, the IgG binding was specific. Abnormally high values on HSA-coated tubes were observed with sera that have been repeatedly frozen and defrozen or stored more than one week at 4°C.

Obviously, the results obtained with grass pollens and DPT prompt to use the plastic tube assay with other allergens. It must be noted however that with some allergen preparation, the material binding to the polystyrene tubes could well not be antigenic.

We have shown that IgG-a-DPT were present in normal sera. Indeed, they could be absorbed on DPT-linked to sepharose and the recovered antibody was shown to inhibit the binding of IgE antibodies to DPT. As this inhibition was not complete, we cannot conclude that all the antigenic determinants present in the DPT extract are simultaneously recognized by IgG and IgE antibodies. The finding of IgG antibodies to DPT or pollens in the serum of normal persons is not new and has already been reported by Holford-Strevens *et al.* (1970), Kawai *et al.* (1972), Romagnani *et al.* (1973), Chapman & Platts-Mills (1978) and Platts-Mills *et al.* (1978). The observations (Romagnani *et al.*, 1973; Delespesse, unpublished data) that DPT extracts stimulate ³H-thymidine uptake in lymphocyte cultures from normal persons further support the concept of a spontaneous immunization of non-allergic people to common pneumallergens. In agreement with the recent data of Chapman & Platts-Mills (1978) we found higher IgG-a-DPT levels in allergic than in non-allergic subjects. However, in opposition to these authors, we did not note a correlation between levels of IgE and IgG antibodies to DPT in sera from untreated patients.

This discordance may be explained by the different purity of allergens used in the two studies. It is likely that, in our assay, we detect some IgG antibodies directed against antigenic determinants absent from their purified fraction, and not recognized by IgE antibodies.

Contrasting with our results on antibodies to DPT, we found a good correlation between IgG- and IgE-anti pollen antibodies in untreated allergic patients. The latter findings are in agreement with the recent report by Platts-Mills *et al.* (1978).

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