

Original article

Phthalic anhydride allergy: development and characterization of optimized hapten–carrier conjugates for improved diagnosis

Background: At present the diagnosis of IgE-mediated hypersensitivity to phthalic anhydride (PA) is based on conjugates that are not characterized or standardized. The aim of this study was to develop optimized and molecularly characterized PA conjugates that can be used to improve the diagnosis of PA-allergy.

Methods: The PA conjugates were synthesized and the number of haptens bound on a carrier protein was estimated by matrix-assisted laser desorption/ionization time of light (MALDI-TOF) mass spectrometry. The ability of conjugates to bind IgE and IgG antibodies was measured by enzyme-linked immunosorbent assay (ELISA). Reactivity of the conjugates *in vivo* was evaluated by skin prick testing.

Results: The most active IgE-binding conjugates had a PA : HSA molar ratio of 80 : 1. In the optimal conjugates the average numbers of PA haptens per carrier molecule of human serum albumin (HSA) were 14–16. In ELISA, all 13 patients and none of the 20 controls had IgE antibodies to optimized PA conjugate. The sensitivity and specificity of the ELISA was comparable to commercial CAP RAST. PA conjugates elicited positive test results in skin prick testing showing that conjugates are immunologically active also *in vivo*.

Conclusions: These results indicate that optimized and molecularly characterized PA–HSA conjugates can be used both *in vitro* and *in vivo* assays to improve the diagnosis of PA allergy.

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Phthalic anhydride (PA) is a low-molecular-weight (LMW) compound industrially used in the production of plasticizers, alkyl and polyester paint resins, and as a curing agent for epoxy resins (1). Exposure to acid anhydrides causes irritation (2) and immunologically mediated respiratory diseases (3, 4). Workers exposed to PA suffering from asthma and rhinitis (5) were first described in 1939 (6). IgE antibodies to PA have been described (7). It has been shown that IgE antibody can be directed either against the phthalyl group itself acting as a hapten, or alternatively against some new antigenic determinants. New antigenic determinants comprise of structural alterations formed after binding of haptens to carrier proteins (8).

The LMW chemicals are not in themselves immunogenic, but after binding with carrier proteins hapten–

protein conjugates emerge, which are capable of inducing specific immune responses (7, 9). Immunologic tests are important for evaluating immediate hypersensitivity to acid anhydrides (10). At present the hapten conjugates used for diagnosis of PA allergy have not been characterized or standardized. Only a few studies show the influence of hapten density on conjugates by their ability to bind to IgE antibodies (8, 11–14). However, none of these studies give direct information of the phthalic anhydride conjugates.

The aim of the present study was 1) to find optimal reaction conditions for the synthesis of PA–protein conjugates, 2) to find characteristic information on the conjugate structures, and 3) to test their ability to bind IgE and IgG antibodies in immunologic assays and to induce skin prick test reactions.

Material and methods

Patients and control sera

The study group consisted of 13 patients who had work-related symptoms attributable to acid anhydride exposure. All patients (Table 1) had positive reactions to in-house PA–HSA conjugates

Abbreviations: ELISA, enzyme-linked immunosorbent assay; Hb, hemoglobin; HD, hapten density; HPLC, high-performance liquid chromatography; HSA, human serum albumin; LMW, low molecular weight; MALDI-TOF, matrix-assisted desorption/ionization time of flight; OA, ovalbumin; PA, phthalic anhydride; PBS, phosphate-buffered saline; PBS–T–HSA, PBS containing 0.05% Tween 20 and 0.2% HSA; RAST, radioallergosorbent test; SDS–PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

Table 1. Characteristics of 13 patients allergic to phthalic anhydride

	Patient number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Age (years)	48	39	42	54	21	50	45	37	52	39	44	32	32
Sex	M	M	F	M	M	M	M	M	F	M	F	F	M
Prick tests (diameter of wheal, mm)													
PA-HSAa (uncharacterized conjugate; 10 mg/ml)	3	*	5	5	6	5	6 ps	4	5	6	4	7	9
Histamine (10 mg/ml)	6	6	6	7	6	7	6	8	8	6	5	6	8
Total IgE (kU/l)	134	279	569	44	203	106	75	82	95	18	13	84	81
Specific IgE CAP RAST (kU/l)	1	13	40.2	5.1	1.4	4.6	9.6	4.8	1	1.5	1	14.9	4.6

HSA: human serum albumin; PA: phthalic anhydride; ps: pseudopodes.

* dermatographism.

(mean wheal diameter 5.3 mm, range 3–8 mm) in skin prick tests, performed as described previously (15). All patients had also positive results in commercial CAP RAST (mean 7.9 kU/l, range 1–40.2 kU/l; Pharmacia). The mean total IgE was 101 kU/l (range 13–279 kU/l). Sera from 10 of these patients were pooled and used for further immunologic studies. Sera from 20 atopic patients with no known exposure to acid anhydrides were used as controls. Four patients (numbers 7, 10, 12, 13 in Table 1) were skin prick tested with different PA-HSA conjugates to analyze reactivity of the conjugates *in vivo*.

Preparation of phthalic anhydride conjugates

Various concentrations of PA (Fluka Chemika, Buchs, Switzerland) dissolved in acetone were added to the cooled solution of 500 µg/ml human serum albumin (HSA, fraction V; Sigma, St Louis, MO) in 50 mM carbonate buffer at pH 9.0. The number of added moles of PA per carrier protein mole was 5, 10, 20, 40, 80, 160, 320 or 640. The mixture was allowed to react on ice for 60 min. The samples were stored at –20°C until the time of analysis. Other proteins, including hemoglobin (Hb; Sigma Chemical Co., St Louis, MO) and ovalbumin (OA; Sigma Chemical Co., St Louis, MO) were tested as carrier proteins.

The conjugates were purified from unreacted components and salts by high-performance liquid chromatography (HPLC) gel filtration. The gel filtration was performed on Fast desalting HR 10/10 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mM carbonate buffer at pH 9.6. The conjugate mixture was subjected to the column at flow rate of 2 ml/min and detection at 280 nm. The eluting peaks were collected and analyzed by sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE; Mini Protean II Cell; Bio-Rad, Hercules, CA). The fractions from the gel filtration were used for ELISA studies.

In order to remove salts which may interfere in mass spectrometric analysis, fractions from the gel filtration were purified by HPLC on a column, ProRPC 5/10 (Pharmacia Biotech, Uppsala, Sweden), using a linear gradient elution with acetonitrile (0–100% in 20 min) in 0.1% trifluoroacetic acid. The flow rate was 0.5 ml/min. The elution was detected at 280 nm, and the fractions collected were analyzed by mass spectrometry.

Mass spectrometric characterization

The extent of hapten incorporation to carrier protein was estimated by MALDI-TOF mass spectrometry. The instrumentation was carried out using a Finnigan MAT Lasermat 2000 (Hemel Hempstead, UK) instrument. About 1 pmol of each purified sample was mixed with 0.5 µl of matrix (33 mM α -cyano-4-hydroxycin-

amic acid in 70% acetonitrile) and air-dried to the sample plate. Laser energy was optimized manually for each sample and the spectrum was recorded from 10 repeated shots. The average number of covalently bound haptens on the protein was estimated from the differences in the measured molecular weights of PA-HSA conjugates vs. nonmodified HSA.

ELISA analysis

Specific IgE and IgG antibodies were measured by ELISA as previously described (16). The Lowry method was used for determination of protein concentrations. PA-HSA conjugate for coating microtiter plates (100 µl/well; Nunc, Roskilde, Denmark) was diluted in 50 mM carbonate buffer to a concentration of 10 µg/ml. The plates were incubated for 3 h at room temperature and overnight at + 4°C. Wells were then postcoated for 1 h with 1% HSA in 50 mM carbonate buffer. After rinsing three times, 100 µl of patient or control serum diluted 1 : 10 with PBS containing 0.05% Tween 20 and 0.2% HSA (PBS-T-HSA) was added to the wells and incubated for 2 h at room temperature. Biotinylated goat antihuman IgE (diluted 1 : 1000 with PBS-T-HSA) or antihuman IgG₁₋₄ (diluted 1 : 1000 for IgG₁, IgG₂ and IgG₄ and 1 : 10000 for IgG₂) (Vector; Burlingame, CA) followed by streptavidin-conjugated alkaline phosphatase (Zymed, San Francisco, CA) diluted 1 : 1000 with PBS-T-HSA was added and incubated for 1 h. After rinsing, a substrate (*p*-nitrophenyl phosphate; Sigma Diagnostics, St Louis, MO) was added and the color was read at 405 nm using an automated ELISA reader (Multiskan MS, Labsystems, Helsinki, Finland). The mean optical density (OD) value plus three standard deviations of the control sera was used as the cutoff limit for positivity.

Skin prick tests

Skin prick tests to PA-HSA conjugates were performed as previously described (15). Histamine hydrochloride (10 mg/ml) was used as a positive control. The PA-HSA conjugates were diluted with Coca solution containing 50% glycerol to a concentration of 500 µg/ml.

Results

Immunologic and biochemical characterization of the PA conjugates

Effect of PA/HSA molar ratio The results showed that the molar ratio of the reacting components had a significant influence on the generated antibody response

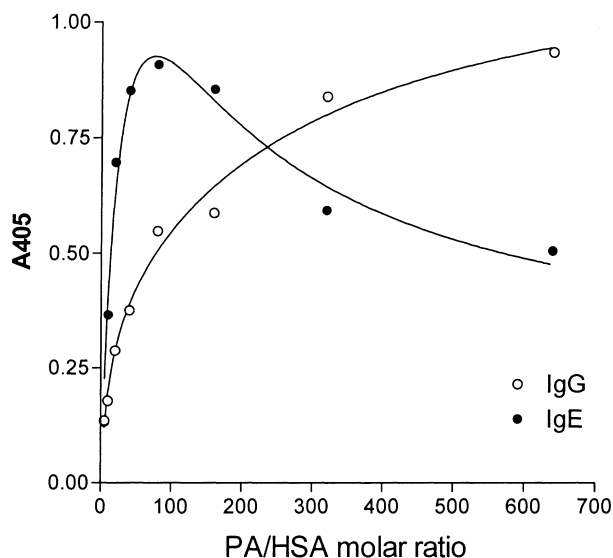


Figure 1. The binding of immunoglobulin IgE (●) and IgG (○) antibodies to phthalic anhydride–human serum albumin (PA–HSA) conjugates in ELISA. The conjugates were prepared at different PA/HSA molar ratios at pH 9.0, using pooled sera.

(Fig. 1). The PA–HSA conjugates with molar ratio 80 : 1 produced the most active IgE-binding conjugate (Fig. 1; solid circles). Binding of IgE was considerably decreased when the PA : HSA molar ratio increased above 80 : 1. In contrast, binding of IgG antibodies to conjugate was progressively increased although the PA : HSA molar ratio increased above 80 : 1 (Fig. 1; open circles).

Effect of different carrier proteins. HSA was more effective carrier protein than ovalbumin or hemoglobin (Fig. 2). In ELISA, IgE binding to hemoglobin or ovalbumin conjugates was approximately 10% of the IgE binding to HSA conjugates (Fig. 2A). The effect of carrier protein to IgG binding was not as critical. Binding of IgG to hemoglobin conjugate was more than 50% of the IgG binding to HSA conjugate (Fig. 2B). Only HSA conjugates were used in further studies.

Hapten density of the conjugates. According to the mass spectrometric analysis an increasing molar concentration of PA to a constant amount of HSA resulted in increased numbers of carrier bound haptens (Fig. 3A). The hapten density (HD) per one carrier molecule ranged from 2 to 29 (Fig. 3B). The ability of conjugates to bind IgE antibodies decreased markedly when the HD range was less than 10 or more than 20. The most reactive IgE-binding conjugates contained 14 haptens per carrier (Fig. 3B).

ELISA analysis with individual patient and control sera

The PA–HSA conjugate (hapten density 14; prepared at pH 9.0 with PA : HSA molar ratio of 80:1) showing strongest IgE-binding ability in pooled sera was further tested with individual patient sera. In ELISA, all 13

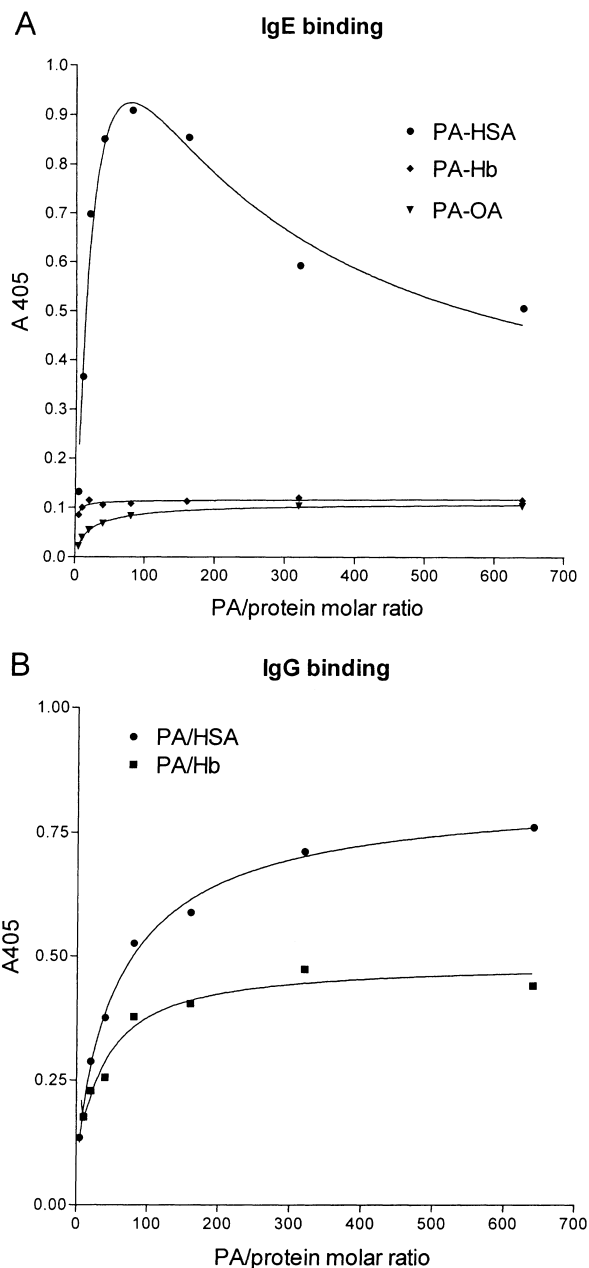


Figure 2. The binding of (A) IgE and (B) IgG to phthalic anhydride (PA)–protein conjugate in ELISA, using pooled sera. Carrier proteins were: HSA, human serum albumin (●); Hb, hemoglobin (■); OA, ovalbumin (▼).

patient sera from PA-allergic patients, but none of the 20 controls, showed IgE-binding to the PA–HSA conjugate (Fig. 4A). The binding of IgG₁₋₄ subclasses to optimized PA–HSA conjugate was also examined in 6 patients (numbers 2, 7, 8, 10, 11, 12) and 10 control sera. Binding of IgG₁ and IgG₄ (Figs 4B and 4E), in sera from PA-allergic patients was significantly elevated compared to controls.

The ELISA results were compared with the results from commercial CAP RAST (Fig. 5). The specific IgE

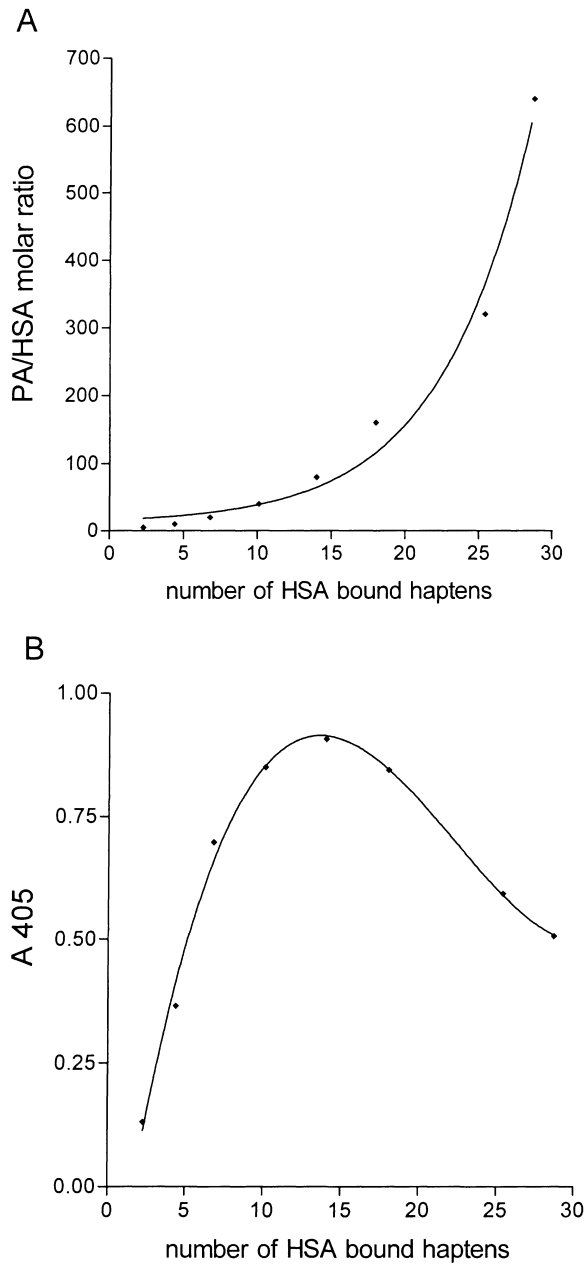


Figure 3. (A) Hapten density of phthalic anhydride-human serum albumin (PA-HSA) conjugates prepared at different PA/HSA molar ratios. (B) Hapten density vs. IgE antibody levels in ELISA. Pooled sera were used.

correlated closely with RAST results ($r = 0.90$). However, five of the patient sera showed slightly or markedly elevated IgE binding in ELISA compared to CAP RAST results (indicated as solid lines in Fig. 5).

Skin prick tests

The main characteristics and skin test results of the four patients are summarized in Table 2. All four patients were positive in skin prick testing. The hapten density

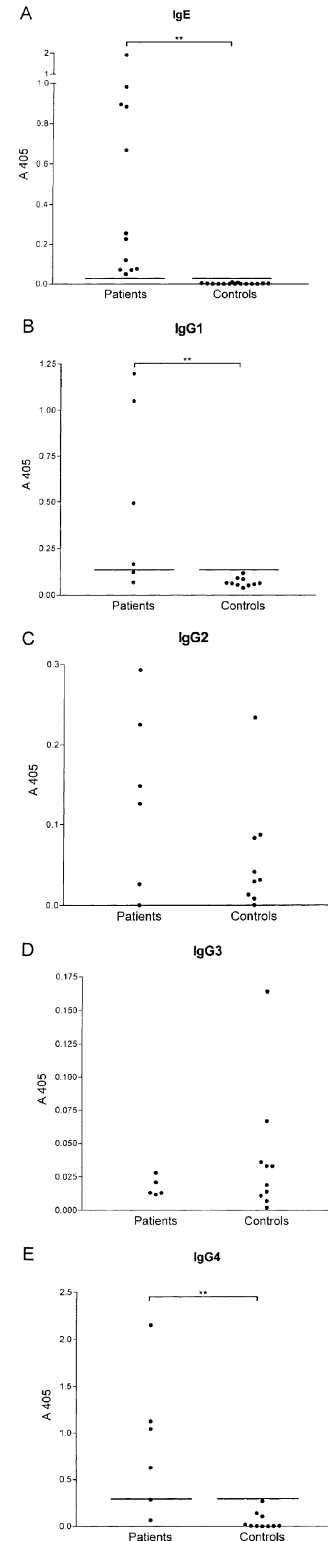


Figure 4. (A) Binding of IgE antibodies to the optimized phthalic anhydride-human serum albumin (PA-HSA) conjugate in sera from 12 patients and 20 controls in ELISA. (B)–(E) Binding of IgG_{1–4} antibodies to the optimized PA-HSA conjugate in sera from 5 to 6 patients and 10 controls in ELISA.

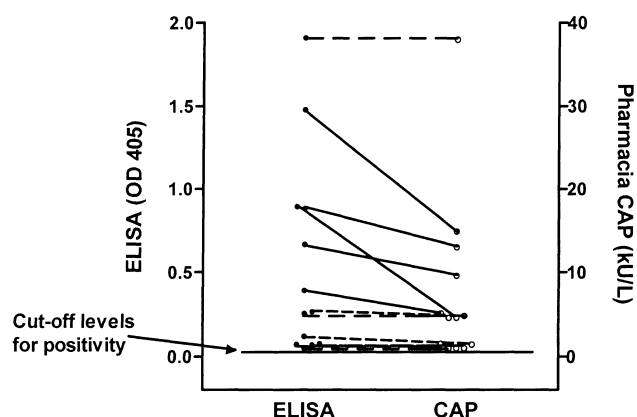


Figure 5. Comparison of IgE antibody levels determined with the optimal phthalic anhydride-human serum albumin (PA-HSA) conjugate in ELISA and commercial CAP method ($r = 0.90$). Patients showing comparable levels of IgE binding to PA conjugates in both assays are indicated as dashed lines; patients showing elevated levels of IgE binding to conjugates in ELISA are indicated as solid lines.

Table 2. Prick test results of four patients allergic to phthalic anhydride

	Patient no			
	7	10	12	13
Former uncharacterized PA-HSA conjugate				
PA-HSA (10 mg/ml)	6**	6	7	9
Histamine (10 mg/ml)	6	6	6	8
Molecularly characterized PA-HSA conjugate				
PA-HSA-HD5 (500 µg/ml)	4	2	5	5
PA-HSA-HD20 (500 µg/ml)	4	6	5	6
PA-HSA-HD80 (500 µg/ml)	7	3	5	7
PA-HSA-HD640 (500 µg/ml)	8	ND	5	6
Histamine (10 mg/ml)	7	7	5	8

HD: hapten density; HSA: human serum albumin; PA: phthalic anhydride.

** Diameter of wheal/mm; ND: not detected.

inducing strongest skin reactivity seemed to vary among patients and was not directly correlated to IgE-binding ability of the conjugates.

Discussion

In the present study the most active IgE-binding conjugates were synthesized by using PA : HSA ratio of 80:1 at pH 9. In this conjugate, the average number of PA units (haptens) per HSA molecule was 14–16. The ability of conjugates to bind IgE antibodies decreased markedly when the HD range was less than 10 or more than 20. These results are in line with the study of Welinder and Nielsen (14) who showed that IgE binding ability of hexahydro- and methyltetrahydrophthalic anhydride conjugates seems to be constant within HD of 10–25.

In previous RAST inhibition studies, PA was showed to form IgE-binding epitopes only if proper carrier proteins were used in the conjugate synthesis (8, 14). This is in line with the present study since the ability of conjugate to bind IgE-antibodies in ELISA was reduced by 90% as compared to HSA conjugates when hemoglobin or ovalbumin were used as carrier proteins. However, the binding of IgG antibodies was reduced only by 40% as compared to HSA conjugates when hemoglobin was used as carrier protein. These results suggest that the binding of IgE to PA conjugate is carrier dependent, whereas binding of IgG antibodies is more hapten dominant. The present results also show that over-substitution of carrier by haptens (HD more than 20 per HSA molecule) reduced the capacity of the conjugate to bind IgE antibodies. In contrast, the binding of IgG antibodies to conjugate was progressively increased although the HD range increased above 20. This suggests that IgG response to hapten conjugates is more polyclonal compared to the IgE response.

These results show that the optimized PA-HSA conjugates used in IgE ELISA functioned at an individual level at least as well as the commercial CAP RAST, and even better in five patients. In addition, binding of IgG₄ and IgG₁ antibodies in sera from PA-allergic patients to optimized PA-HSA conjugates were significantly elevated compared to controls. Whether PA-specific IgG₁ and IgG₄ antibodies play a role in the pathogenesis of PA-allergy, or whether these specific antibodies can be used as a marker of the disease, will be evaluated in further studies. The ELISA method used in this study offers well-known advantages over radioimmunoassays: it is less time consuming, equipment and reagent expenses are markedly less, and it is free from the hazards of radioisotopes (8, 17).

The wheal diameters induced by PA-HSA conjugates analyzed here were slightly smaller compared than those induced by former uncharacterized PA conjugates. This may be because the former conjugate concentration (10 mg/ml) used in skin prick testing was 20 times higher than that used in the present study (500 µg/ml). Hapten densities inducing the highest skin reactivity seemed to vary across patients and were not directly correlated to the ability of the conjugates to bind IgE. This suggests that PA conjugate optimized for immunologic *in vitro* assays (e.g. for ELISA) may not necessarily be an ideal reagent for skin prick testing. For optimal skin prick test reactivity, the use of a pool of standardized and molecularly characterized PA conjugates with different hapten densities may be required.

Diagnosis and testing of immediate-type allergies to chemicals are challenging compared to other allergies, as binding of the chemical hapten to the carrier protein is routinely needed for immunogenicity prior to testing (15). Such complexes are usually created by trial and error (15). This study describes a systematic approach to optimize test materials. The sensitivity and specificity of

the *in vitro* and *in vivo* assays used in diagnosis of immediate allergy to PA can be improved by increasing the quality of the test materials. Studies are also needed to improve diagnostic tests of immediate allergy to other chemicals in our environment, including the workplace.

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