

Serum albumins are the major site for *in vivo* formation of hapten-carrier protein adducts in plasma from humans and guinea-pigs exposed to type-1 allergy inducing hexahydrophthalic anhydride

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Summary

Background Organic acid anhydrides (OAAs) are highly allergenic compounds used in the chemical industry. The OAAs probably act as haptens but the proteins that form conjugates with OAAs *in vivo* are still unknown. Conjugates between the anhydrides and serum albumins (SAs) have routinely been used when testing for OAA-specific antibodies. However, the use of SA as the carrier-protein in these tests has never been evaluated.

Objective The aim of this study was to identify major and also immunologically relevant protein conjugates of a particularly sensitizing OAA, hexahydrophthalic anhydride (HHPA), in plasma.

Methods Plasma was obtained from a HHPA-exposed worker, from a guinea-pig (GP) exposed to HHPA in an exposure chamber for 2 weeks (8 h/day, 5 days/week) and from a GP exposed once, nose-only, to tritium-labelled HHPA for 8 h. The plasma was fractionated using ion exchange chromatography and gel filtration. These fractions and also aliquots of unfractionated plasma were hydrolysed, derivatized and analysed for anhydride adduct content using gas chromatography–mass spectrometry. Further, plasma from the tritium labelled HHPA-exposed GP was separated by SDS gel electrophoresis and analysed by autoradiography. In addition, immunologically relevant proteins were identified through specific IgE and IgG immunoblottings using sera from exposed workers.

Results For humans > 85% and for GPs > 74% of the HHPA-adducts coeluted with SA in plasma. Autoradiography of GP-plasma shows a single 66 kDa protein that binds HHPA. IgE immunoblotting shows a major 66 kDa and a minor 28 kDa protein which could be inhibited by HHPA-SA conjugate. IgG immunoblotting showed a major 66 kDa protein and several minor protein bands.

Conclusion This study shows SA to be the major protein in plasma that forms adducts *in vivo* with HHPA. The results also show that in an *in vitro* synthesized HHPA plasma protein conjugate, HHPA-specific IgE and IgG antibodies bind preferably to the SA.

Keywords: organic acid anhydrides, hexahydrophthalic anhydride, type-1 allergy, plasma, protein adducts, serum albumin, ion exchange chromatography, gel filtration, autoradiography, immunoblotting

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Table 1 Characteristics of the studied workers

	Subject					
	1	2	3	4	5	6
Sex	Male	Male	Male	Female	Female	Male
Work task	Founding	Founding	Founding	Finishing	Finishing	Repairing
U-HHP acid ($\mu\text{mol/mol}$)	ND	80	10	330	ND	3100
TPPA-level (pmol/mL)	13.8	0.25	0.16	1.0	0.06	8.3
Exposure time (years)	7	6	0.6	20	20	24
Smoking	Yes	Former	No	Former	Former	No
Atopy (prick test)	No	No	No	No	No	No
Asthma	No	No	No	Yes	No	No
Rhinitis	No	No	No	Yes	Yes	Yes
FVC (%)	108	95	104	109	113	116
FEV 1.0 (%)	105	83	100	72	115	118
Methacholin test	NHR	NHR	NHR	NHR	ND	NHR
Specific IgG (OD)	1.45	1.34	1.23	1.12	0.21	0.02
Specific IgE (%)	0.4	28.5	4.4	22.4	27.8	0
Total IgE (kU/L)	18	13	109	18	161	8

U-HHP acid = urinary levels of HHP acid adjusted for creatinine (gives a measure of the exposure during 1 day, $90 \mu\text{mol/mol}$ creatinine is approximately equivalent to an air level of $10 \mu\text{g HHPA/m}^3$ [10]); ND = not determined; TPPA = total plasma protein adducts (gives a measure of the exposure over several weeks); FVC (%): = forced vital capacity (% of expected); FEV 1.0 (%) = forced expiratory volume in 1 s (% of expected); NHR = not hyperreactive; OD = optical density [15]; RAST value [15].

Introduction

The prevalence of diseases such as airway disorders, allergic asthma and rhinitis is on the increase and it is estimated that between 2% and 5% of asthma in industrialized nations is occupationally induced, due to exposure to aeroallergens [1]. Organic acid anhydrides (OAAs) are a family of reactive, low molecular weight compounds which are widely used in the chemical and plastic industry as crosslinking agents. Earlier studies have shown that more than 60% of OAA-exposed workers may develop airway symptoms such as asthma and rhinitis [2,3] even at exposures as low as less than $20 \mu\text{g/m}^3$ [4]. Allergy of type-1 according to the classification by Gell and Coombs [5] seems to be a probable mechanism in many of the cases, as specific immunoglobulin (Ig) E against the *in vitro*-formed conjugates between the anhydrides and human serum albumin (SA) has been found in about half of the symptomatic workers [4]. The relevance of the IgE-antibodies for symptoms has been shown by nasal challenges of exposed workers using OAA-SA conjugates [6,7]. Thus, the OAAs probably act as haptens, conjugating *in vivo* with endogeneous proteins to form complete antigens, but the identity of these

proteins is still unknown. Such information is important not only for understanding the mechanisms behind induction of allergies but also for development of diagnostic tools, e.g. conjugates between SA and anhydrides have been used routinely in testing for OAA-specific antibodies ever since the 1970s [8] but the use of SA as the carrier-protein in these tests has never been evaluated. Thus, there may be other choices of carrier-proteins that are more relevant.

Here we study one particular sensitizing anhydride, hexahydrophthalic anhydride (HHPA) [9], and its conjugation products in plasma. The aim of the study was to identify the major *in vivo*-formed HHPA-protein adduct and also immunologically relevant plasma protein conjugates of HHPA, in occupationally exposed workers and in guinea-pigs (GPs) exposed in an exposure chamber. In order to achieve this, we separated the plasma using ion-exchange chromatography (IEC) and gel filtration and analysed the anhydride adducted proteins using gas chromatography-mass spectrometric (GC-MS) methods. We identified the proteins in plasma that form adducts with HHPA using gel electrophoresis and autoradiography. Further, we identified immunologically relevant proteins by immunoblot methods.

Materials and methods

Exposed workers

The workers investigated were employed at a plant manufacturing electrical capacitors. They were exposed to HHPA mainly by inhalation of the vapours but some contribution from dermal exposure cannot be ruled out. However, it has been shown that the percutaneous absorption of HHPA is low [10]. The time-weighted average air levels (personal sampling) of HHPA in the plant ranged from <1 – $420 \mu\text{g}/\text{m}^3$. Characteristics of the studied workers are shown in Table 1.

Animal exposure to unlabelled HHPA

An inbred, white male GP of Dunkin-Hartley strain, weighing about 300 g (Sahlins, Malmö, Sweden) was exposed to $1.3 \text{ mg HHPA}/\text{m}^3$ in a 70-L glass chamber. The gaseous HHPA was generated by a permeation technique as previously described [11]. The animal was exposed whole-body, 5 h/day, 5 days/week for 2 weeks. It was maintained on the facilities with food and water provided *ad libitum* and on a cycle of 8 h light and 16 h dark, but during exposure in the chamber it had access only to water. Immediately after the last exposure the animal was anaesthetized by an intramuscular injection ($1 \text{ mL}/\text{kg}$ body weight) of a mixture of ketamine hydrochloride ($50 \text{ mg}/\text{mL}$; Park-Davis, Barcelona, Spain) and xylazine chloride ($20 \text{ mg}/\text{mL}$; Bayer, Gothenburg, Sweden), prepared in a ratio of 3 : 2. Blood was collected and processed as described below. The animal was killed by an overdose of ketamine/xylazine.

Animal exposure to [^3H]HHPA

[$^3\text{H}_2$]HHPA was synthesized as described elsewhere [12]. The radioactive HHPA atmosphere was generated by passing a dry air flow of $0.2 \text{ L}/\text{min}$ through a vial with solid [$^3\text{H}_2$]HHPA. The airflow was administered through a Teflon tube close to the nostril of a GP kept anaesthetized by continuous administration of the ketamine hydrochloride and xylazine chloride mixture, in a 2-L air-tight glass chamber. Humidified air with a flow of $2 \text{ L}/\text{min}$ was passed into the exposure chamber and the outlet air was passed through a scrubber containing NaOH solution. The animal was exposed for 8 h, after which blood was collected as described below. The exposure level was $0.5 \text{ mg}/\text{m}^3$ ($3 \mu\text{mol HHPA}/\text{m}^3$) with a specific activity of $34 \text{ GBq}/\mu\text{g}$ ($0.2 \text{ GBq}/\mu\text{mol}$) HHPA.

Ethics

The human study was approved by the ethics committee at Lund University and all workers gave an informed consent.

The animal studies were approved by the Animal Research Committee at Lund University.

Collection of biological samples

Blood samples from workers were drawn from an antecubital vein and the blood from GPs was obtained by heart puncture. For collection of sera, the blood samples were collected in glass test tubes and allowed to clot at room temperature for 1 h. The tubes were stored at 4°C overnight, after which they were centrifuged and sera separated. For collection of plasma, the blood was collected in 10 mL Venoject® blood sampling tubes (Terumo Europe, Leuven, Belgium) containing sodium heparin. This blood was allowed to cool to room temperature and then centrifuged at 1500 g for 10 min for the separation of plasma. All samples were stored in polyethylene tubes at -20°C .

Dialysis

One millilitre of plasma was dialysed in dialysis tubing (SpectraPore, Spectrum, Houston, TX, USA; $2.0 \text{ mL}/\text{cm}$; cut-off 12–14 kDa) against phosphate buffered saline containing 2 mM EDTA (PBS-EDTA) at 4°C over 6 days with stirring. The buffer was changed after 8 h and then on days 2, 3 and 5.

IEC of proteins in plasma

The plasma (1 mL) was dialysed as described above and then diluted 50 : 50 with 20 mM ethanolamine buffer (pH 9.5). Aliquots of the sample ($500 \mu\text{L}$) were injected via a 1-mL loop into an ÄKTA purifier liquid chromatography system (Pharmacia Biotech, Uppsala, Sweden) fitted with a Mono Q HR 5/5 column. (Pharmacia Biotech). The mobile phase was 20 mM ethanolamine (pH 9.5) with a flow rate of $1.0 \text{ mL}/\text{min}$. A gradient from 0 to 100% 1 M NaCl in the mobile buffer was applied in three stages. First 0–20% 1 M NaCl in five column volumes (1 column volume = 0.98 mL), then from 20 to 40% 1 M NaCl in 20 column volumes and finally from 40% to 100% 1 M NaCl in five column volumes where it was kept for four column volumes. One-millilitre fractions were collected, dialysed and analysed for SA adduct content using GC-MS as described below. An aliquot of the non-fractionated plasma was also analysed for total plasma protein adduct content for recovery calculation purposes.

An agarose gel electrophoresis screen was run on the fractions (see below). Using the screen, the fractions containing the majority of SA were identified, selected and pooled. To obtain enough material for subsequent analysis, fractions from a number of runs on the column were usually combined. The pooled sample was dialysed as described

above and at the same time reduced in volume using polyethylene glycol, MW 20000 (PEG) in the dialysis buffer (50 mg PEG/L PBS-EDTA buffer). The concentrated SA sample was further purified using gel filtration as described below.

Further purification of SA fractions using gel filtration

Dialysed fractions from the IEC containing SA were further concentrated, first by Centriprep (Amicon, Danvers, MA, USA) and then by Centricon (Amicon). Aliquots of 100 µL of the concentrated samples were injected into the ÄKTA purifier LC system equipped with a Superdex 200 column (model 10/30; Pharmacia Biotech). The mobile phase was 0.05 M NH_4HCO_3 with a flow rate of 0.5 mL/min. One-millilitre fractions containing proteins (fractions 5–13) were collected. To obtain enough material for quantification, corresponding fractions from a number of runs on the column were combined. These fractions were dialysed and concentrated in PBS-EDTA buffer with PEG (50 mg PEG/L PBS-EDTA buffer) to about 2 mL. The fractions were thereafter quantified for adducts of HHPA and an agarose gel electrophoresis screen (see below) was also run on the fractions.

Agarose gel electrophoresis screen

Agarose gel (0.8%) prepared in barbital-sodiumbarbital buffer (0.075 M, pH 8.6, 2 mM EDTA), as described previously [13], was used to separate and identify proteins in plasma and in fractions from IEC and gel filtration.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Acrylamide gel (4–15%) in tris/glycin buffer (Biorad, Hercules, CA, USA) was used for SDS-PAGE.

Quantification of adducts of HHPA

The levels of HHPA bound to proteins in plasma were determined as described elsewhere [14]. Briefly, the plasma samples were dialysed to remove free acids. The HHPA was then hydrolysed from the proteins to the corresponding acids which were purified using a solid phase extraction procedure and derivatized by pentafluorobenzyl bromide. The obtained esters were analysed by GC-MS in the negative ion chemical ionization mode.

Synthesis of HHPA-plasma protein conjugate

The conjugates were synthesized, purified and stored as described previously [14]. Thus, HHPA (61.5 mg) was

dissolved in 0.5 mL dioxane and then slowly added to 10 mL human plasma at room temperature. The pH was adjusted to 8.0 using aqueous NaOH and the resulting solution was stirred overnight at 4 °C. The conjugate was purified by dialysis (Spectrapor; cut-off 3.5 kDa) against PBS-EDTA buffer during 5 days and stored at –20 °C.

Autoradiography of plasma from GP exposed to radioactive HHPA

The plasma from the GP exposed to radioactive HHPA was dialysed against PBS-EDTA for 2 weeks at 4 °C. An aliquot of the plasma containing 80 cpm was separated by SDS-PAGE. The gel was stained with Coomassie blue, destained and then treated with EN³HANCETM autoradiography enhancer (Dupont, Boston, MA, USA). The gel was washed in 10% glycerol in water, wrapped in cellophane film, dried and exposed on CEA RP X-ray film between intensifying screens at –70 °C for 15 months.

Specific IgG immunoblotting

Three aliquots (approximately 2 µg) of the HHPA-plasma protein conjugate were separated by SDS-PAGE. Non-conjugated plasma as a control was also separated adjacent to each of the three conjugate lanes. The proteins were blotted to a preconditioned PVDF membrane (Biorad) using a Semi-dry Electrobloater A (Ancos, Denmark) at 150 mA for 3 h. The transfer buffer contained 48 mM Tris, 39 mM Glycin, 20% methanol and 1.3 mM SDS. The membrane was then blocked by incubation in 5% dried milk in Tris Buffer Saline Tween (TBST; 10 mM Tris, 150 mM NaCl 0.25% Tween 20, pH 8.0) for 2 h at 21 °C. The blocked membrane was then cut in three pieces, each with one conjugated and one non-conjugated plasma blot. The blots were incubated for 2 h at 21 °C, each in a different serum (diluted 1 : 20 with 5% dried milk in TBST) from three different workers known to have high levels of specific IgG in their sera. A gel with both conjugated and non-conjugated plasma was run and stained with Coomassie-blue for identification of SA band. The serum-incubated membranes were washed three times with 5% dried milk in TBST and incubated, for detection purposes, with an alkaline phosphatase conjugated rabbit anti-human IgG (D0336; Dako, Glostrup, Denmark) diluted 1 : 1700 with 5% dried milk in TBST, at 21 °C for 2 h. The membranes were then finally washed with TBST and developed by Nitro Blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

IgE immunoblotting

Two sets of three inhibition mixtures were prepared by addition of either 3.0, 0.15 or 0 mg of a conjugate between human SA and HHPA [15] to 1 mL serum from two

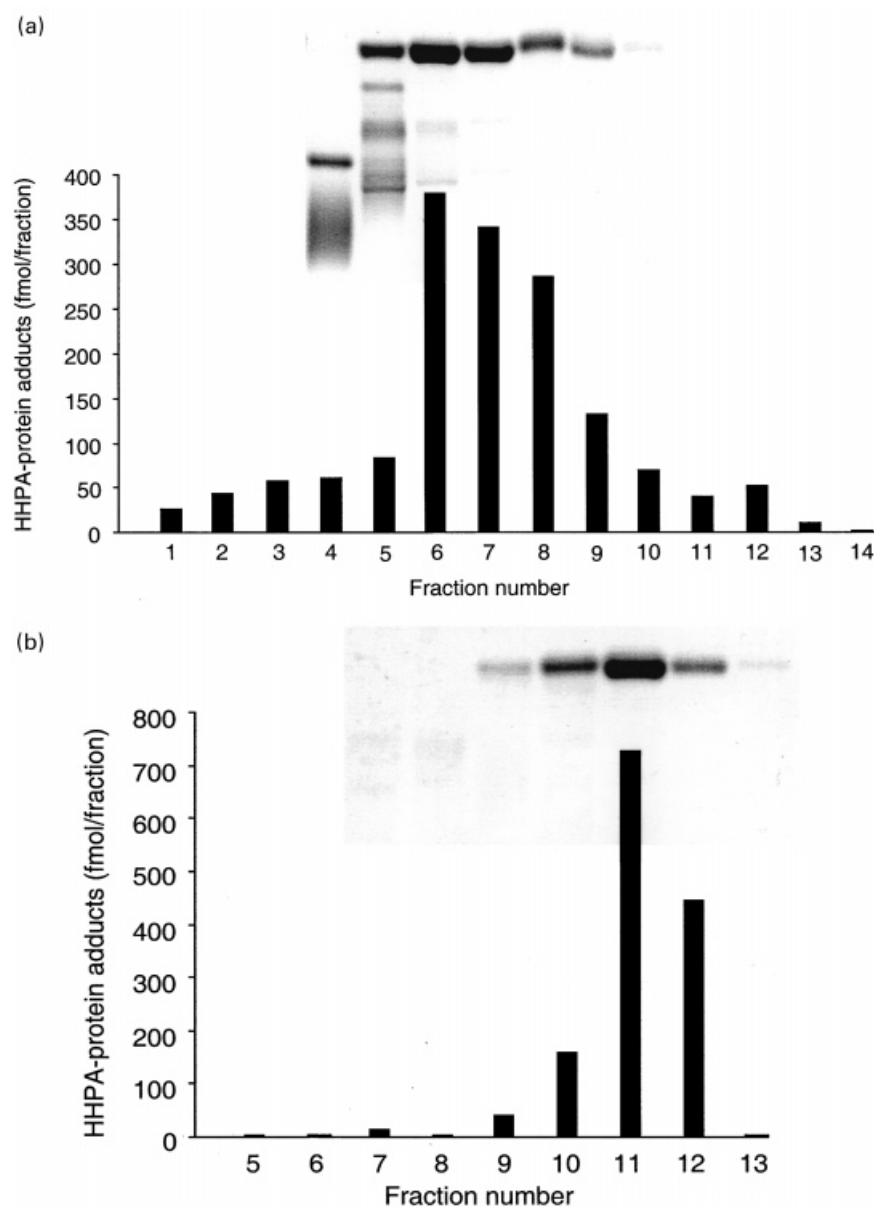


Fig. 1 (a) Amounts of HHPA adducts hydrolysed from plasma proteins in fractions collected after ion exchange chromatography using a Pharmacia Mono Q HP 5/5 column. The plasma was from a worker exposed to about 340 μg HHPA/ m^3 . In addition, an agarose electrophoresis screen of the proteins in the fractions is included. The adducted proteins show a typical delayed elution compared with serum albumin which originates in the conversion of a positively charged amino group in lysine to a negative carboxylic group from the anhydride. (b) Amounts of HHPA adducts in different protein fractions after separation on a Pharmacia Superdex 200 gel filtration column. The proteins fractionated were the serum albumin-containing fractions from the anion exchange chromatography separation in (a). The coelution of the HHPA adducts and serum albumin is shown by an agarose electrophoresis screen of the proteins in the fractions.

HHPA-exposed workers known to have HHPA-specific IgE. These mixtures were incubated at 4 °C overnight, centrifuged and then diluted 1 : 40 with 5% dried milk in TBST. Two sets of three aliquots (approximately 2 μg) of HHPA-plasma protein conjugate were separated on SDS-PAGE gels, blotted to membranes and blocked with milk as described above for IgG. Non-conjugated plasma as a control was also separated adjacent to each of the three conjugate lanes. Both membranes were divided in three, each with a conjugated and a non-conjugated plasma lane and incubated in the different inhibition solutions for 2 h at 21 °C. These were thereafter washed three times with 5% dried milk in TBST. For detection purposes they were

incubated with an ^{125}I -labelled rabbit/mouse monoclonal anti-human IgE (Pharmacia, Uppsala, Sweden) diluted to an activity of about 700 kcpm with 5% dried milk in TBST-buffer at 21 °C for 2 h. After a final wash with TBST, the membranes were wrapped in cellophane and exposed on CEA RP X-ray film (CEA group, Strängnäs, Sweden) between intensifying screens at -70 °C for 8 days.

Results

Conjugates between HHPA and proteins in human plasma

Fractions from four IEC runs of plasma from an

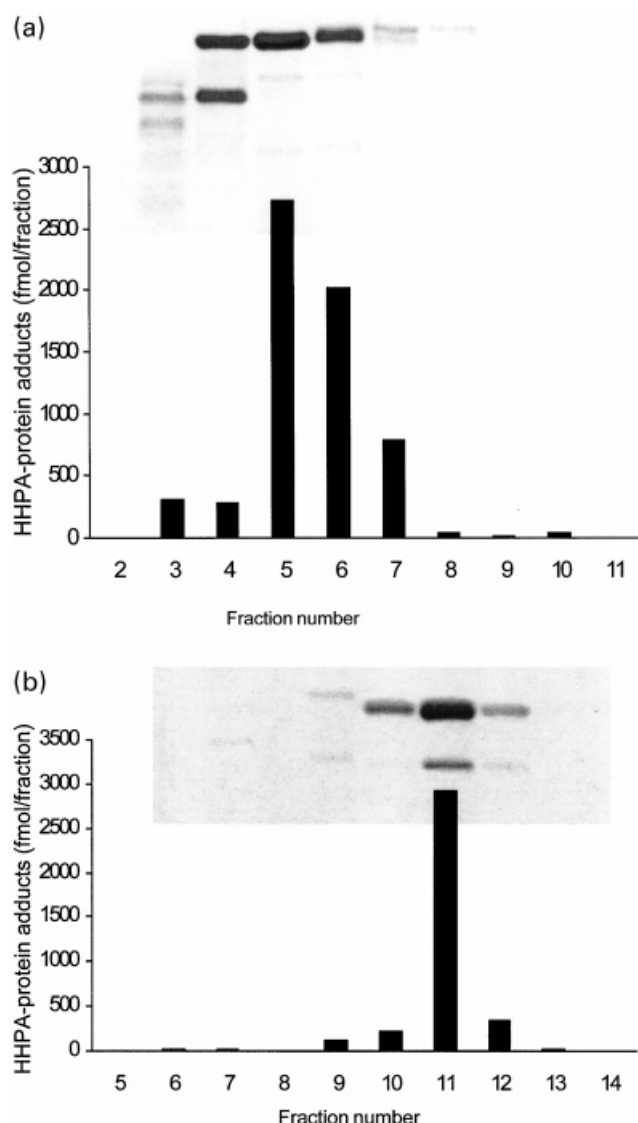


Fig. 2 (a) Amounts of HHPA adducts hydrolysed from plasma proteins in fractions collected after ion exchange chromatography using a Pharmacia Mono Q HP 5/5 column. The plasma was from a guinea-pig exposed to about 1300 μg HHPA/ m^3 . In addition, an agarose electrophoresis screen of the proteins in the fractions is included. The adducted proteins show a typical delayed elution compared with serum albumin which originates in the conversion of a positively charged amino group in lysine to a negative carboxylic group from the anhydride. (b) Amounts of HHPA adducts in different protein fractions after separations on a Pharmacia Superdex 200 gel filtration column. The proteins fractionated were the serum albumin-containing fractions from the anion exchange chromatography separation in (a). The coelution of the HHPA adducts and serum albumin is shown by an agarose electrophoresis screen of the proteins in the fractions.

HHPA-exposed worker (Subject 6) were collected. Corresponding fractions from two runs were pooled and analysed for adduct levels. In Fig. 1(a), the amounts of HHPA-protein adducts in the various IEC fractions are shown. The agarose electrophoresis screen on the fractions is also presented in the same figure, showing the pattern of elution of plasma proteins in the different fractions. The eluted amount of HHPA-adducts in all 14 fractions was 99% of the totally injected amount of adducts on the IEC column. The majority of the HHPA-adducts, 88% of the totally eluted amount of adducts, was recovered in fractions 5–10, coeluting with SA. Further, fractions 5–14, containing the SA, from the two remaining IEC runs were pooled into one sample. The sample was fractionated using gel filtration and the corresponding fractions from six runs were pooled. These fractions were investigated for HHPA adduct content. In Fig. 1(b), the amounts of HHPA-protein adduct in the various gel filtration fractions are shown with the corresponding agarose electrophoresis screen showing the plasma protein bands. The eluted amount of HHPA adducts in all nine fractions was 108% of the totally injected amount of adducts on the gel filtration column. The majority of the HHPA adducts, 98% of the totally eluted amount, was recovered in fractions 9–12, coeluting with SA.

Conjugates between HHPA and proteins in GP plasma

Seven aliquots of plasma from a GP exposed to HHPA was fractionated using IEC. A total of eight fractions were analysed for HHPA adducts. In fractions 4–8, coeluting with SA, 96% of the totally eluted HHPA adduct was recovered. The amount of HHPA protein adduct in all collected fractions was 84% of the totally injected amount of adducts (Fig. 2a). Fractions 4–8 from four of the runs on IEC were pooled and aliquots were fractionated using gel filtration. The fractions containing SA were analysed for HHPA adduct content. In fractions 9–12, coeluting with SA, 93% of the totally eluted HHPA-protein adduct was recovered (Fig. 2b). The recovery of HHPA adducts from all fractions was 103% of the totally injected amount of adducts on the column.

Autoradiography of GP plasma

Plasma from the GP exposed to gaseous, radioactive HHPA was separated by SDS-PAGE and analysed by autoradiography. An autoradiogram as well as a Coomassie blue stained gel are shown in Fig. 3. A single prominent band is seen at about 66 kDa in both cases.

Immunoblotting for specific-IgG

The synthesized HHPA-plasma protein conjugate and

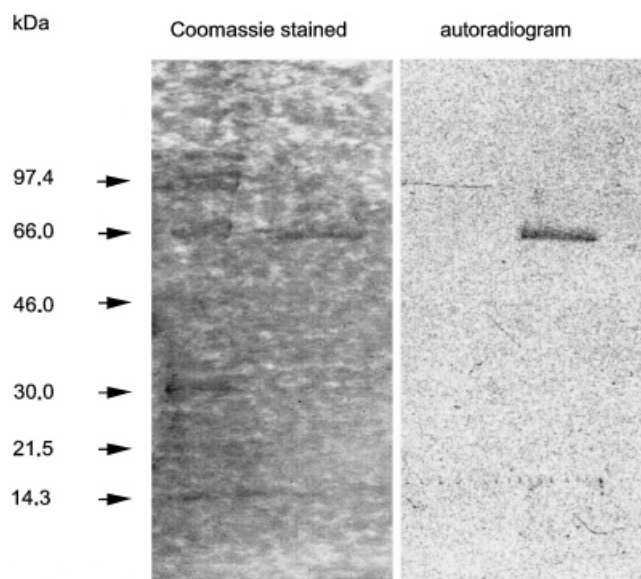


Fig. 3 Autoradiogram of plasma proteins from a guinea-pig separated by SDS-PAGE. The guinea-pig was exposed to gaseous, tritium-labelled HHPA for 8 h and the plasma was collected at the end of exposure. Following the electrophoresis the plasma proteins were exposed on an X-ray film for determination of radioactivity. The autoradiogram shows a single radio-labelled band at the same position as serum albumin in the Coomassie Blue stained gel.

non-conjugated plasma were separated on a SDS-PAGE gel and transferred to PVDF membrane. The immunoblotting was performed on three aliquots of the conjugate using sera from three HHPA-exposed workers known to have specific IgG antibody (Subjects 1–3). The results are

shown in Fig. 4. A dominant band is seen at 66 kDa in the HHPA-conjugate lane for all three workers. The corresponding bands in the non-conjugated plasma lanes are very weak. There are diffuse bands at about 50 kDa in the HHPA-conjugate lanes which are also present in the non-conjugated plasma. There are weak bands at about 28 and 80 kDa in the HHPA-conjugate lanes but these are absent in the non-conjugated plasma lanes. Some smaller proteins seen in the Coomassie Blue stained gel (e.g. at 25 kDa) are absent in the blottings.

Immunoblotting for specific-IgE

The HHPA-plasma protein conjugate and the non-conjugated plasma were run on SDS-PAGE gels, transferred to membranes and immunoblotted using sera from two HHPA-exposed workers known to have HHPA-specific IgE. The IgE in these sera had previously been inhibited to various extents. The results are shown in Fig. 5. For both workers, there is one major band at about 66 kDa in all three conjugate lanes, decreasing in intensity with increasing amount of inhibition of specific IgE in sera. There are also diffuse bands at about 28 kDa which could be inhibited by HHPA-HSA conjugate. There are no bands in the non-conjugated plasma lane and no further bands appeared even after exposure on the film for a further month.

Discussion

The results from this study show that the major HHPA-adduct forming protein *in vivo* in plasma is SA. The results

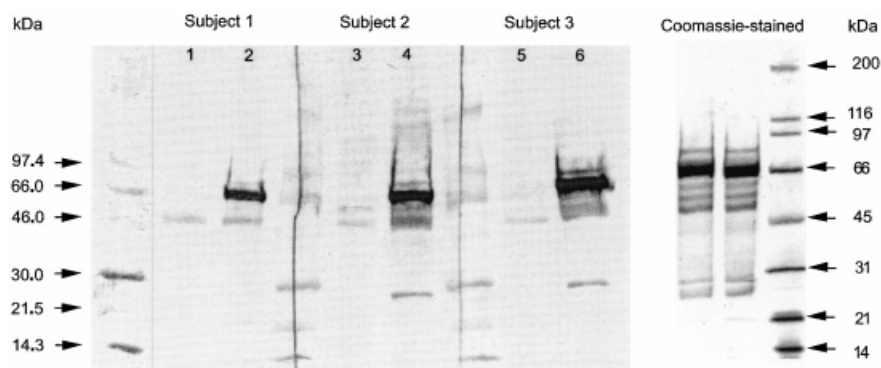


Fig. 4 Immunoblotting of HHPA-specific IgG antibodies using sera from three different HHPA-exposed workers (Subjects 1–3). A conjugate between HHPA and proteins in a plasma sample obtained from a blood bank was synthesized and separated alongside a sample of non-conjugated plasma using SDS-PAGE. The Coomassie Blue stained gel shows no difference compared with the non-conjugated plasma. The proteins were blotted to a PVDF membrane which was then incubated with sera from the exposed workers and with anti-human IgG antibody. The blottings were developed using Nitro Blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP). In all three workers most of the HHPA-specific IgG antibodies were bound to serum albumin (lanes 2, 4 and 6). However, some other proteins, but not all, also bound specific IgG. In the non-conjugated plasma only some binding was seen, probably from the IgG (lanes 1, 3 and 5).

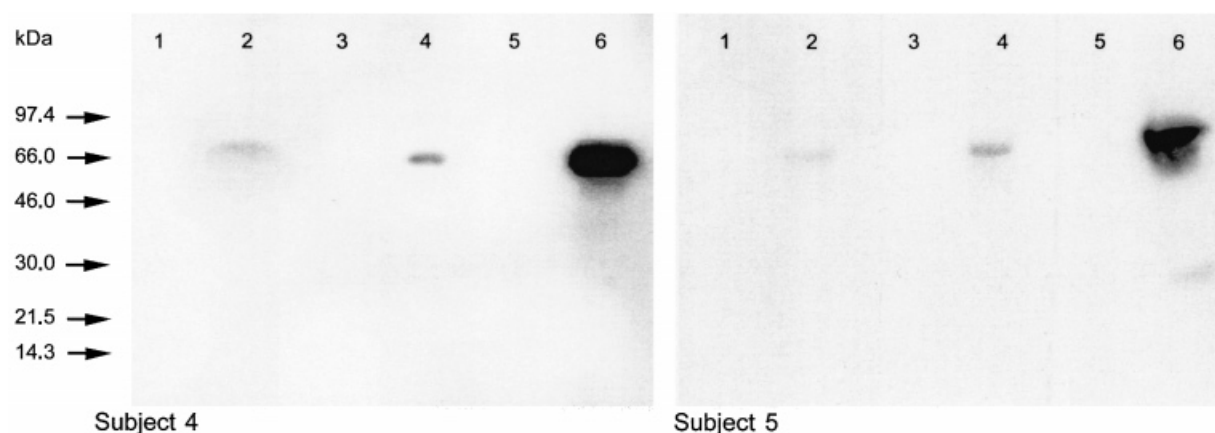


Fig. 5 Immunoblotting of HHPA-specific IgE antibodies using sera from two different HHPA-exposed workers (Subjects 4 and 5). Before blotting, the sera were inhibited with HHPA-SA conjugate; 3.0 (lanes 1 and 2), 0.15 (lanes 3 and 4) and 0 mg/mL (lanes 5 and 6). A conjugate between HHPA and proteins in plasma from a blood bank was synthesized and separated (lanes 2, 4 and 6) alongside a sample of non-conjugated plasma (lanes 1, 3 and 5) using SDS-PAGE. The proteins were thereafter blotted to a PVDF membrane which was then incubated with inhibited plasma from exposed workers and an ^{125}I labelled anti-IgE antibody (Amersham Pharmacia). The membrane was exposed on an X-ray film for determination of radioactivity.

also suggest selective binding where the HHPA-specific IgE and IgG antibodies bind preferably to SA in an *in vitro* synthesized HHPA-plasma protein conjugate.

Studies of adduction of low molecular weight allergens to endogenous proteins is important for understanding how these chemicals induce airways disease. Furthermore, such information is important for development of new diagnostic tools for determination of specific antibodies. SA-conjugates with OAAs have been routinely used in tests for specific IgE and IgG antibodies but the use of the SA has never been evaluated. In this study, as a first step in identification of immunologically relevant proteins conjugating with OAAs as well as in evaluation of SA as carrier-protein in antibodies tests, we chose to focus on adduction of HHPA to plasma proteins.

In a previous study from our group [14] we showed that HHPA-adducts coeluted with SA in an IEC separation of plasma from 10 HHPA-exposed workers. However, the crude separation of the protein in that study is insufficient for identification of SA as the adducting protein. In this study we used a much more robust protein purification strategy. We first separated the plasma using IEC and then applied a second protein purification technique, gel filtration, on the fractions containing the SA. We also analysed the collected fractions individually for presence of HHPA-adducts.

Our present results from the IEC and gel filtration studies of the plasma from exposed workers as well as GPs show that at least 74% of the HHPA-adducts are conjugated with SA. Considering the fact that SA makes up for only about 45–55% of the proteins in plasma and yet over 74% of the HHPA-adduct is with SA, this clearly indicates a rather

specific binding of HHPA to SA compared with the other plasma proteins. Another interesting point to note is that the conjugated SAs were seen to elute slightly later compared with native SA in the IEC. This is what would be expected as the binding of HHPA to SA has an effect on the ionic charge of the protein, giving it a more negative state.

The extensive protein purification used in the studies where adducts in fractions were quantified using GC-MS is very time-consuming and thus plasma from only one worker and one guinea-pig was analysed. However, the results from the previous study [14] where samples from 10 workers exposed to varying levels of HHPA were analysed for protein adducts seem to be in agreement with the present ones. In addition, the results from the autoradiography in this study indicate SA to be the major adducting protein in GPs.

The presence of HHPA-adducted SA in plasma is interesting. HHPA is a highly reactive chemical with a half-life in water of less than 1 min [16]. Lindh *et al.* [12] showed that in GPs and rats HHPA does not seem to reach the alveolar regions. It is unclear whether HHPA may survive crossing the membranes and reach the blood stream in the upper respiratory airways. One possible explanation may be that HHPA forms a masked reactive conjugate with an endogenous compound in the airways lumen which then may cross the membranes into the blood stream where it can react with SA. Formation of masked reactive conjugates has been described for toluene diisocyanate and glutathione [17].

In the immunoblotting studies the main protein bands in the conjugate lane binding both IgE and IgG are in the region of 66 kDa, corresponding to the molecular weight of

SA. In the non-conjugated plasma lane, in the IgE study, this band is absent and in the IgG study there is only a very weak band, probably due to a non-specific binding. The fact that the 66 kDa band could be inhibited by the addition of HHPA human SA-conjugate in the IgE inhibition test, is also convincing evidence for the specific antibody affinity to the conjugate. In the IgE immunoblotting study only one other protein band seemed to bind HHPA-specific IgE. This band is much weaker than the SA band and this could either be because the HHPA-specific antibodies have lower affinity for this protein conjugate or simply because these proteins are a minority in plasma compared with SA. This binding could be inhibited by an HHPA-HSA conjugate. This suggests that it is the hapten, and not any new protein determinants formed from binding of the HHPA, which is important for the inhibition.

In the IgE immunoblotting study, no other band appeared even after prolonged exposure on the film. In the IgG immunoblotting study, however, there were some more bands in the conjugate lanes which were not present in the non-conjugated plasma lanes, suggesting that IgG bind also to other plasma protein conjugates besides the SA conjugate. Conversely, there are bands in the Coomassie Blue stained lanes which do not appear in the conjugate lanes, hinting that there might also be plasma proteins that do not bind HHPA or that the HHPA-specific antibodies show no affinity for these conjugates.

From this study we may conclude that SA is the major adducting protein in plasma but the adduct levels in plasma are very low. At exposure levels that seem to sensitize a large fraction of the workers ($< 20 \mu\text{g}/\text{m}^3$), less than one out of one million SA is adducted [14]. It may be questioned whether these adduct levels are sufficient to induce an immunological response. On the other hand, the levels of HHPA-SA conjugates may be higher in, for example, the airways and administration of HHPA-SA conjugate to rats has been shown to induce production of HHPA-specific antibodies [18]. However, there may of course be other more critical protein bindings, from an immunological point of view, giving rise to antibodies and allergic diseases. Lindh *et al.* [12] found extensive binding of inhaled radioactive HHPA to epithelial cells in the upper airways region in GPs and rats. In addition, binding of radioactivity was found in the alimentary tract and in the eyes. Conjugates of hexamethylene diisocyanate and proteins from the airways epithelium have shown immunological activity [19]. From the present result it may also be concluded that compared with other plasma proteins, SA seems to be a relevant choice of carrier-protein in the tests for specific antibodies. However, binding to proteins not found in plasma could still be a possible explanation for symptomatic workers testing negative for specific antibodies

as analysed by the present methods for ELISA and RAST. These questions will be addressed in our further investigations.

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