

Letter

Determination of chemical-specific IgGs in serum by an enzyme-linked immunosorbent assay with partial peptides of human serum albumin

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ABSTRACT — Many different types of chemicals are used in industry, and occupational allergies are becoming a serious problem in the field of industrial hygiene. In this study, we employed a novel enzyme-linked immunosorbent assay (ELISA) with partial peptides of human serum albumin (HSA) to quantify chemical-specific immunoglobulin G (IgG) in serum for evaluating exposure to chemicals. When HSA partial peptides containing lysine residues were mixed with formaldehyde (FA) or phthalic anhydride (PA), almost all lysine residues were lost. Mass spectrometry revealed that PA and FA formed imine and tertiary amine, respectively, with lysine residues in the peptides. Thus, we used FA- or PA-peptide adducts as an artificial antigen to detect FA- and PA-specific IgGs in serum. The concentrations of FA- and PA-specific IgGs in workers at plants utilizing plastic resins were significantly higher than those in general subjects. This method can estimate exposure levels to chemicals and thus be expected to contribute to the diagnosis of allergies in workers and to the prevention of health hazards due to harmful chemicals.

Key words: Chemical-specific IgG, ELISA, Carbonyl compounds

INTRODUCTION

Increasing evidence shows that occupational allergies, such as dermatitis and asthma, could be due to chemicals used for producing plastic polymers; occupational allergies have become a serious problem in the field of industrial hygiene (Bernstein, 1997). Patch test and skin prick tests are commonly used to diagnose causative chemicals for allergic dermatitis. However, these tests have the risk of sensitizing patients (Hillen *et al.*, 2003), and alternative diagnoses are therefore essential. In general, low-molecular-weight compounds are considered haptens and

thus cannot elicit an immunoreaction, although reactive substances can bind to carrier proteins to trigger immunogenicity. For instance, several kinds of chemical-protein adducts have been identified in the human body, such as adducts with human serum albumin (HSA), hemoglobin, cytokeratin 18, glucose-regulated protein, actin and tubulin (Day *et al.*, 1996; Lange *et al.*, 1999; Wisnewski *et al.*, 2000; Hettick and Siegel, 2011). Therefore, measurement of chemical-specific immunoglobulin G (IgG) and/or immunoglobulin E (IgE) in the blood is considered an effective approach for diagnosing exposure to chemicals and occupational allergies without sensitization risks.

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Because blood concentrations of IgG are much higher than those of IgE, specific IgG can be a more useful diagnostic marker of occupational allergy than IgE considering the sensitivity of the measurement. In fact, specific IgE has been shown to identify inhalation challenge-positive workers with 14%-31% sensitivity and 89%-97% specificity, while the corresponding sensitivity and specificity estimates for specific IgG were reported to be 46%-72% and 74%-92%, respectively (Bernstein *et al.*, 2006).

Several methods to detect chemical-specific IgG in the blood have been developed. In particular, measurements targeting toluene-2,4-diisocyanate (TDI) have been widely investigated as diagnostic markers, and enzyme-linked immunosorbent assays (ELISAs) with TDI-HSA adducts have been used as a substrate challenge (Park *et al.*, 2002). An anti-TDI haptenated protein monoclonal antibody was reported as a tool for biomonitoring of TDI exposure (Ruwona *et al.*, 2011), and this antibody was also applied to sandwich ELISAs to detect aromatic diisocyanates adducts (Lemons *et al.*, 2013). An ELISA using formaldehyde (FA) and phthalic anhydride (PA)-HSA adducts as a substrate was also reported (Dykewicz *et al.*, 1991; Nielsen *et al.*, 1988). We previously reported a dot blotting-based method for the detection of chemical-specific IgGs, including TDI, FA and PA (Tsuji *et al.*, 2016; Kawamoto *et al.*, 2015).

ELISAs and dot blotting methods that have been reported for the detection of chemical-specific IgGs employ full-length HSA or other proteins as carriers to produce artificial antigens. Chemicals can react with multiple amino acids in the full-length carrier proteins. For instance, TDI is reported to react with 37 amino acids, including lysine, aspartic acid and glutamine, in HSA (Hettick *et al.*, 2012). Thus, multiple antibodies with different epitopes are detected if full-length carrier proteins are used as an artificial antigen, which could be one reason why it is difficult to determine the relationships between chemical-specific antibodies and occupational allergies.

ELISA is convenient for several diagnoses because it can analyze many samples with a brief period. In this study, we aimed to construct an ELISA for determination of chemical-specific IgGs in sera using an HSA partial peptide having a limited reactive amino acid residue as an antigen. This ELISA is expected to detect single chemical-specific antibodies, which could have advantages in exactly estimating exposure levels to chemicals as well as in reducing measurement error. The carbonyl compounds FA and PA were selected for specimens, as large amounts of FA and PA have been used in industry and FA and PA reportedly elicit occupational allergies (Fowler *et al.*, 1992; Wernfors *et al.*, 1986). We designed

an HSA partial peptide containing lysine residues because carbonyl compounds can bind to amino groups through a nucleophilic addition reaction. The structure of chemical-peptide adducts was revealed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), and the ELISA was tested with serum samples from general subjects and workers in the plastics industry.

MATERIALS AND METHODS

Preparation of chemical-HSA peptide complexes

Two kinds of biotin-conjugated HSA partial peptides were synthesized and purified to the > 95% level at Eurofins Genomics (Tokyo, Japan): HSA Peptide Lys1: Biotin-ALLVRYTKKVPQVSTP-COOH (MW = 2026.45) and HSA Peptide Lys2: Biotin-ERQIKKQTALVELV-COOH (MW = 1881.24). FA and PA were dissolved in DMSO to a final concentration of 100 mM. HSA partial peptides were dissolved in PBS to a final concentration of 5 mM. Peptides were mixed with FA or PA in 100 mM sodium carbonate buffer, pH 10.8, or phosphate buffer, pH 8.0, respectively, with final concentrations of 0.1 mM peptide and 10 mM FA or PA. The mixture was incubated for 1 hr at 37°C. After centrifugation, the supernatant was used as a chemical-peptide adduct.

Measurement of the amino groups

The reaction of 2,4,6-trinitrobenzenesulfonic acid (TNBS) with primary amino groups within peptides was performed as previously described (Tsuji *et al.*, 2016). The chemical-peptide adducts (25 μ L) were mixed with 50 μ L of 4% NaHCO₃, followed by the addition of 50 μ L of 0.01% TNBS. The reaction was carried out at 42°C for 2 hr and then was stopped by the addition of 25 μ L HCl. The absorbance was measured at 340 nm. Known concentrations of L-lysine were used as standards.

MALDI-TOF MS analysis

The chemical-peptide adducts were diluted 10-fold in distilled water, and 0.5- μ L samples were added on the sample plate. Then, 0.5 μ L of 10 mg/mL 2,5-dihydroxy benzoic acid (in 0.1% trifluoroacetic acid: acetonitrile = 3: 2 solution) was plated. After air drying, each sample was analyzed using an AXIMA QIT MALDI TOF mass spectrometer (Shimadzu, Kyoto, Japan). All spectra were acquired in the positive linear mode. Mass calibration was performed using two internal standards, angiotensin II ((M+H)⁺ at m/z 1046.5423) and ACTH fragment18-39 ((M+H)⁺ at m/z 2465.1989), with the ProteoMass Peptide and Protein MALDI-MS Calibration

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Kit (Sigma-Aldrich, St. Louis, MO, USA).

Study subjects

Worker subjects (N = 10) were recruited at a company handling plastic resins (plastic resins mainly used: phenolic resins, polyester resins, polyurethane resins) in Kyushu, Japan from 2013 to 2015. Their mean age was 39.1 years old and the average length of service was 11.5 years. None of the subjects handled plastic resins outside the workplace. General subjects (unexposed during work, N=10) were recruited in Kyushu in 2014. Their mean age was 46.4 years old. The study was approved by the Ethics Committee of the University of Occupational and Environmental Health in 2013 (H25-008).

Serum preparation

After the collection of blood samples, the blood was allowed to clot for approximately 3 hr and was then centrifuged at $2,500 \times g$ for 30 min to separate the serum portion of the blood. The sera were transferred to clean tubes and stored at -80°C until use.

Enzyme-linked immunosorbent assay (ELISA)

One hundred microliters of HSA peptide Lys1 or 2 at a final concentration of $5 \mu\text{M}$ were added to a streptavidin-coated 96-well plate (IMMOBILIZER, Thermo Scientific, Waltham, MA, USA), and the plate was incubated for 1 hr at room temperature. The same concentration of biotin was used as a blank control. FA or PA was added to the wells at a final concentration of 0.5 mM, followed by incubation for 1 hr at 37°C . The plate was blocked with protein-free blocking buffer (Pierce), and then human serum was added to each well (1/100 dilution for FA and 1/1,000 dilution for PA). After 3 hr of incubation at room temperature, HRP-conjugated anti-human IgG was added to the wells for 1 hr. Next, 1-Step Ultra TMB-ELISA Substrate Solution (Thermo Scientific) was added to the wells, and then the reaction was stopped with sulfuric acid. The absorbance at 450 nm was measured to determine the chemical-specific IgGs in serum. Human IgG (Human IgG, Whole Molecule Control, Thermo) was biotinylated using the Biotin Labeling Kit-NH2 (DOJINDO LABORATORIES, Kumamoto, Japan) according to the manufacturer's instruction and then used as a standard.

Statistical methods

Two-group comparisons were performed using Mann-Whitney U test. All analyses were performed by the Bell-Curve for Excel (Social Survey Research Information, Tokyo, Japan), and statistical significance was assumed

when $p < 0.05$ (two-sided).

RESULTS AND DISCUSSION

Modulation of HSA partial peptide by chemicals

FA and PA can react with lysine residues with high pKa values in proteins because of their nucleophilic carbonyl groups. HSA contains 59 lysine residues with different reactivity with nucleophiles. Hettick *et al.* (2012) reported that 5 of the lysine residues in HSA, Lys351, Lys413, Lys414, Lys524 and Lys525, were highly reactive with the vapor phase of toluene isocyanate. Therefore, we focused on 2 series of lysine (Lys413-414 and Lys524-525) to design 2 peptides, HSA peptide Lys1 and HSA peptide Lys2 (See Materials and Methods).

We first investigated the number of lysine residues in peptides after reaction with FA and PA. The number of amino groups in HSA peptide Lys1 or HSA peptide Lys2 was 55.7 ± 1.2 and 62.7 ± 8.2 nmol/mg peptide, respectively, indicating that almost all lysine residues were free in each peptide (Fig. 1). When FA and PA were mixed with HSA peptide Lys1 and HSA peptide Lys2 under basic conditions, the lysine residues of both HSA peptides were almost completely lost (Fig. 1). Therefore, lysine residues in the peptides were modified by the reaction with FA or PA. To identify the chemical structure of the reactants, we next measured the molecular mass of the chemical-HSA peptide adducts by MAL-

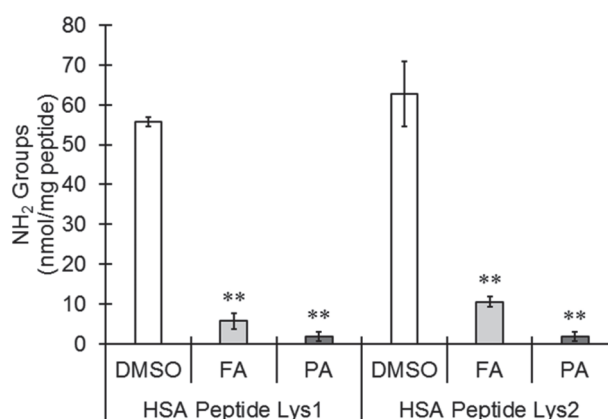


Fig. 1. Decreases in amino groups in HSA peptides after reaction with FA or PA. HSA peptide Lys1 and Lys 2 were reacted with FA and PA to form chemical-peptide adducts. The primary amino groups of HSA peptide were determined using TNBS. The values represent the means \pm S.E.M. of 4 separate experiments. P-values were obtained using Mann-Whitney U tests. **P < 0.01 vs. the DMSO-treated group.

DI-TOF MS. Comparing the MS spectra of HSA peptide Lys1 with that of its FA adducts, the peaks of the FA adducts were approximately 24 m/z higher than those of unreacted peptide (Fig. 2A). The adducts with HSA peptide Lys2 and FA also showed 24 m/z higher peaks than unreacted HSA peptide Lys2 (data not shown). Therefore, two imines could be produced by the reaction of the carbonyl groups in FA with the primary amino group in HSA peptide Lys1 (Fig. 2C). The peaks of the PA adducts of HSA Peptide Lys1 were higher by approximately 130 m/z than those of unreacted HSA peptide Lys1 (Fig. 2B), and almost the same shift was observed in the case of HSA peptide Lys2 reacted with PA (data not shown). Based on these MS analyses, tertiary amines could be produced by nucleophilic attack of primary amino groups against the carbonyl group of PA (Fig. 2C). The TNBS measurement showed that almost all amino groups reacted with PA (Fig. 1); however, peaks derived from the unreacted HSA peptide Lys1 were observed in the sample of PA-HSA peptide Lys1 adducts. Thus, PA adducts might be degraded by the laser at the step of peptide ionization to generate the original peptide.

Determination of chemical-specific IgGs in human serum

We collected serum from general subjects and workers at plants utilizing plastic resins, and we then determined the concentrations of FA- and PA-specific IgG in sera by ELISA using HSA peptides. HSA peptide Lys1 or Lys2 was bound to the bottom of the plate through a streptavidin-biotin interaction. The lysine residues of the peptides were modified by adding FA or PA to the well to produce artificial antigens on the plate, and then the serum was applied to each well to determine the amount of chemical-specific IgG in the serum (Fig. 3A). Based on control human IgG challenges, the detection limit of this ELISA was 0.1 ng/mL antibody in serum. Anti-FA-HSA peptide Lys1 and anti-FA-HSA peptide Lys2 antibodies were detected in the serum of both general subjects and workers (Fig. 3B and C). However, the levels of anti-FA-HSA peptide Lys1 antibody in the serum of workers were significantly higher than those of the general subjects (Fig. 3B). No difference was detected in the levels of anti-FA-HSA peptide Lys2 antibody between the general subjects and the workers (Fig. 3C). The amount of anti-PA-HSA peptide Lys1 antibody in the serum of the workers was significantly higher than that of general subjects, but there was no significant difference in the levels of anti-PA-HSA peptide Lys2 between the 2 groups (Fig. 3D and E). Interestingly, the concentration of antibodies for HSA peptide Lys1 was much higher than that

for HSA peptide Lys2, suggesting that modification of HSA peptide Lys413-414 with chemicals showed robust immunogenicity and that the antibody for HSA peptide Lys1 can reflect exposure more effectively against chemicals than that for HSA peptide Lys2. Taken together, our ELISA with partial HSA, especially HSA peptide Lys1, could measure chemical-specific IgG in serum with high sensitivity.

General subjects also may have come into contact with these chemicals in daily life because FA and PA are used as adhesives, lacquers and hardeners, which might be the reason why antibodies for anti-FA HSA peptide and anti-PA-HSA peptide were detected in the serum of the general subjects. Because the workers are exposed to FA and PA due to their usage of FA and PA in the workplace, they showed high concentrations of IgGs specific for FA and PA in serum, indicating the useful application of the ELISA developed here for evaluation of FA and PA exposure. Several workers with high levels of FA- or PA-specific antibody showed allergic symptoms (data not shown). Therefore, this ELISA might be useful for the diagnosis of occupational allergies.

Several studies have detected chemical-specific IgG by ELISA; in these studies, full-length HSA or other proteins were used as a carrier (Dykewicz *et al.*, 1991; Nielsen *et al.*, 1988). However, in such cases, it is difficult to evaluate the relationships between the concentration of IgG and industrial allergies, as detected IgGs constitute a mixture of different IgGs. Using the ELISA developed in this study, chemical-HSA peptide can be immobilized onto the bottom of a 96-well plate in the same direction because of the streptavidin-biotin interaction, which can contribute to higher reproducibility. Importantly, the advantage of this ELISA is that it can detect a single chemical-specific antibody, which could be useful for understanding the relationships between serum chemical-specific IgG concentrations and allergy in industry. The next step is to examine the relationships between occupational allergy and the IgGs that were measured in this study.

Industrial chemicals other than FA and PA react with specific amino acids in protein; for instance, methyl acrylate, which is a material for acrylic fiber and acrylic resin, strongly reacts with cysteine (Böhme *et al.*, 2009). The HSA molecule includes 35 cysteine residues, but only Cys34 is free, whereas the others form disulfides. Therefore, a partial HSA peptide containing Cys34 might be used for the evaluation of exposure to methyl acrylate. Again, this novel ELISA with HSA peptide can be applied to other chemicals besides FA and PA to evaluate exposure and occupational allergies.

In conclusion, a novel peptide ELISA was established

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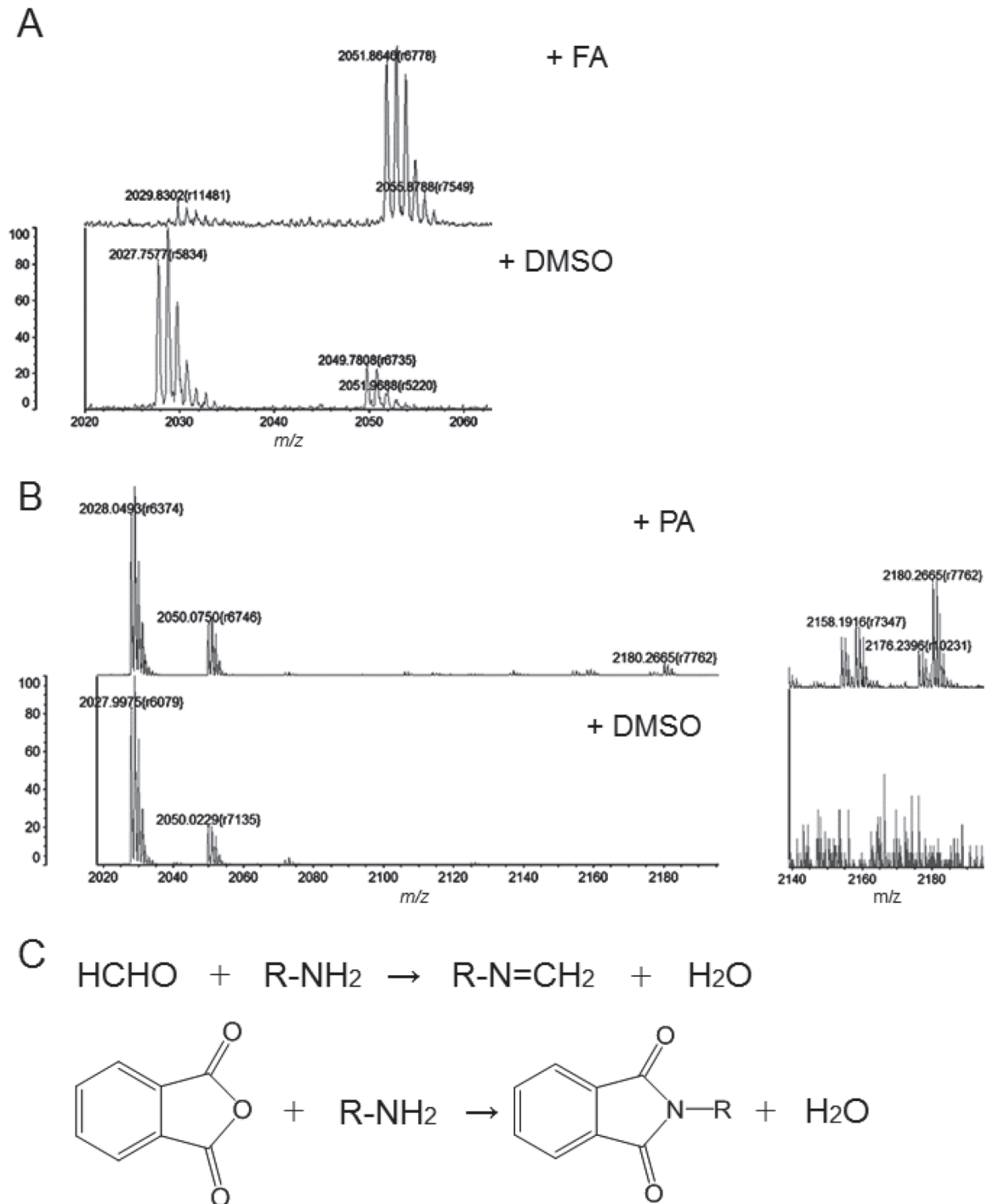


Fig. 2. Identification of the chemical structure of FA- and PA-HSA peptide adducts by MALDI-TOF MS analysis. HSA peptides were reacted with FA and PA and then subjected to MALDI-TOF MS analysis. Panels A and B show the typical MALDI-TOF MS spectra of HSA peptide Lys1 reacted with FA or PA, respectively. The right picture is enlarged view of PA spectra ranging from 2140 to 2195. Panel C shows the putative reaction formula of the primary amino group with FA or PA.

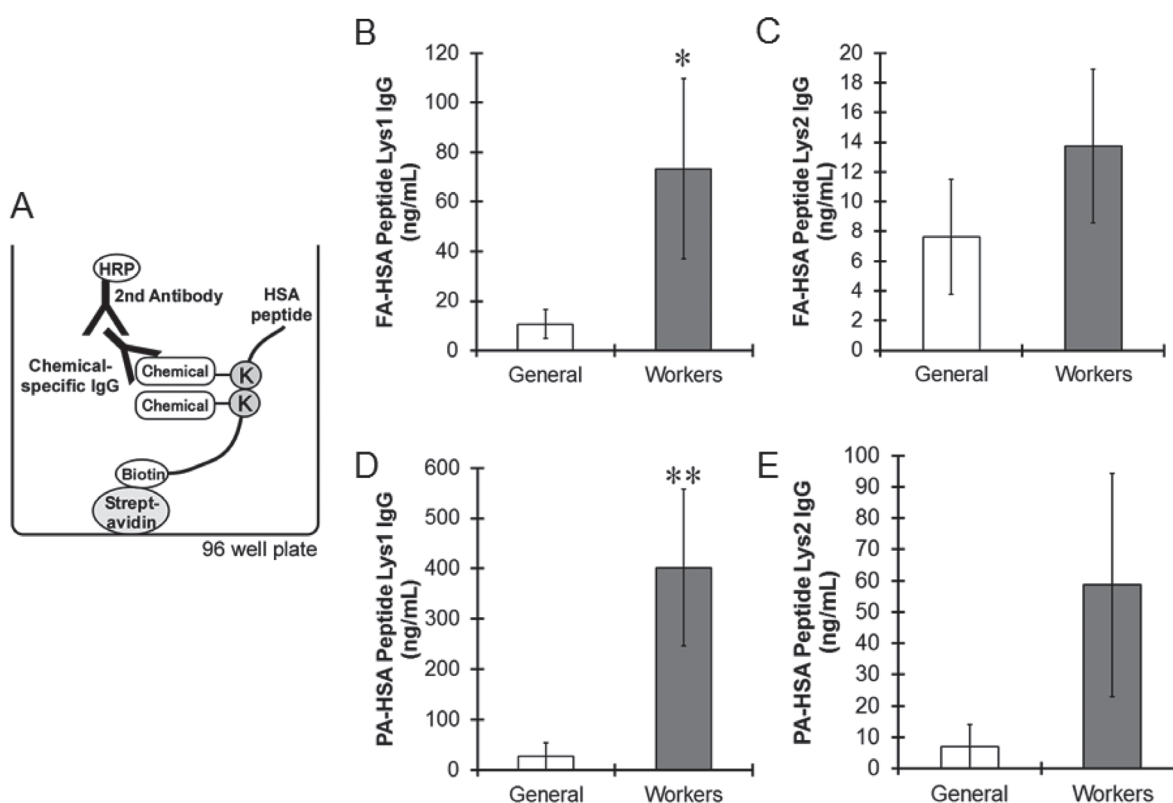


Fig. 3. Comparison of chemical-specific IgG concentrations in the serum of general subjects and industrial workers. Panel A shows a schematic of the ELISA using HSA partial peptides. HSA peptides were reacted with FA or PA and then interacted onto the bottom of plate via the formation of the streptavidin-biotin complex. Chemical-specific IgGs which can bind to FA or PA-HSA peptide adducts were detected by the addition of secondary anti-human IgG antibody conjugated with HRP, followed by chemiluminescence detection. Biotinylated control human IgG was used as a standard. Panels B to E show the concentration of chemical-specific IgG in the serum of general subjects (General) or workers in plants utilizing plastic resins (Worker). The values represent the means \pm S.E.M ($n = 10$ subjects in each group). P-values were obtained using Mann-Whitney tests. * $P < 0.05$, ** $P < 0.01$ vs. general subjects.

for the detection of chemical-specific IgG in serum. This method can estimate exposure levels to chemicals and thus be expected to contribute to the diagnosis of allergies in workers and to the prevention of health hazards due to harmful chemicals.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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