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Diverse Profiles of Specific IgE Response to Toluene Diisocyanate (TDI)-Human Serum Albumin Conjugate in TDI-Induced Asthma Patients

The prevalence studies on specific IgE to toluene diisocyanate (TDI)-human serum albumin (HSA) conjugate in TDI-induced asthma have shown variable results. In this study, we attempted to compare specific IgE bindings to TDI-HSA conjugate and its specificity using 3 different conjugates. Sera were collected from 20 TDI-induced asthma and 10 controls. Specific IgE were measured by ELISA using three TDI-HSA conjugates; two from Carnegie Mellon (CM; 98 and 99 CM conjugates) and one from Ajou University. To evaluate specificity and cross-reactivity, ELISA inhibition tests were applied. Positive and negative predictive values between Ajou conjugate and 98 CM conjugate were 75% and 100%. Those between Ajou and 99 CM were 100% and 93.8%. One patient showed an isolated positive response to the Ajou with negative responses to the other two conjugates. ELISA inhibition test using this patient's serum revealed the significant inhibitions by the Ajou and minimal inhibitions by the others. On the other hand, another patient showed an isolated positive response to 99 CM with negative responses to the others, and ELISA inhibition test showed significant inhibition by 99 CM with minimal inhibitions by the others. These results suggest that specific IgE bindings to a new antigenic determinant of TDI-HSA conjugate can be heterogeneous and differ from one individual to another.

Key Words: *Specific IgE; TDI-HSA Conjugate; Epitopes; Antigenic Determinant; Population Characteristics*

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INTRODUCTION

Isocyanate chemicals are currently the most common causes of occupational asthma (OA) throughout the world (1). Among workers exposed to various types of diisocyanates, i.e. toluene diisocyanate (TDI), methylene diphenyldiisocyanate (MDI), and hexamethylene diisocyanate (HDI), TDI is the most common cause of OA in Korea (2) with a prevalence of 2.9% to 13% in exposed workers (3, 4).

Although considerable controversy remains regarding the pathogenesis of TDI-induced asthma, several groups of investigators have suggested that TDI acts via immunological mechanism. Indeed, serum-specific IgE antibodies have been identified in sensitized workers with a prevalence ranging from 0 to 50%, and some authors have reported a specificity of nearly 100% (5-8).

TDI is a highly reactive chemical, which undergoes changes in its the tertiary structure when conjugated with human serum albumin (HSA). Studies on antigenic determinants on TDI-HSA conjugates, to which specific IgE binds, have indicated that heterogeneous populations of IgE may be present in the patients' sera (9, 10). Specific IgE may develop against TDI itself, or against linkage sites between TDI and HSA, or against neo-antigens generated by modification due to interactions with TDI. Furthermore, degrees of substitution may exert influence on the binding of specific IgE with TDI-HSA conjugates. Actually, TDI-HSA conjugates at lower degrees of substitution were found to bind specific IgE more readily than TDI-HSA conjugates at higher degrees of substitution in RAST assays (11). Our previous study (7) showed individual differences in specific IgE bindings according to the preparatory method of TDI-HSA conjugate.

In this study, in order to confirm the individual differences and specificity of specific IgE bindings, we compared specific IgE bindings by enzyme-linked immunoabsorbent assay (ELISA) using three kinds of TDI-HSA conjugates; two from the laboratory of Carnegie Mellon

University, U.S.A. (CM conjugates, 98 CM and 99 CM) and one from Ajou University (Ajou conjugate). These three conjugates were selected as the most sensitive ones among many pools of TDI-HSA conjugates prepared at each University. ELISA inhibition tests were done to confirm specificity of IgE bindings and to evaluate cross-reactivity between the different conjugates.

MATERIALS AND METHODS

Subjects

Twenty subjects with TDI-induced asthma, whose diagnosis of OA had been confirmed by positive responses to bronchoprovocation tests to TDI (TDI-BPT), were enrolled. They received anti-asthmatic medications according to symptom severities. Unexposed age-matched healthy subjects were enrolled as a control group. Sera from all subjects were collected at diagnosis; all of them had stopped using inhaled or oral steroids at least 4 weeks before the study. They underwent an interview, chest radiography, skin prick test with common inhaled allergens, lung function measurement, and inhalation challenge with both methacholine and TDI (80:20=2.4 form: 2.6 form, Aldrich, U.S.A.). All subjects gave their informed consent and the protocol was accepted by the Institutional Review Board of Ajou Medical Center, Suwon, Korea.

Bronchial challenge test with methacholine and TDI

The methacholine bronchial challenge test was done according to the method described previously (8). Briefly, aerosols were generated by a DeVilbiss 646 nebulizer connected to a DeVilbiss dosimeter driven by compressed air (DeVilbiss Co., Doylestown, PA, U.S.A.). Five inhalations of normal saline at 5 min intervals were followed by a series of successively doubled doses of methacholine (0.075-25 mg/mL) until a 20% fall in forced expiratory volume in one second (FEV1) was observed or until the maximum dose was given. FEV1 was measured 5 min after the beginning of each set of inhalations of aerosolized methacholine. The methacholine PC_{20} level was determined by interpolation from the dose-response curve. The TDI bronchial challenge test was performed according to a standardized protocol (8). Briefly, the subjects were exposed to pure TDI monomers (Aldrich, U.S.A.) in a small closed room for 5-15 min until asthmatic symptoms were induced. The concentration of TDI measured by TLD-1, a toxic gas detector with Cheakey (HSA Scientific, Lincolnshire, IL, U.S.A.), was 20 ppb. FEV1 and forced expiratory flow rate ($FEF_{25-75\%}$) were measured with a spirometer (MultiSPIRO SX/PC, Pempe, AZ, U.S.A.) immediately before exposure and every hour for 8 hr after the exposure.

Preparation of TDI-HSA conjugates

2, 4-TDI-HSA Ajou conjugate was prepared as described previously (7, 8) by using a modification of Tse and Pesce's method. 2, 4-TDI (2, 4 g) was added to 90 mL of 1% HSA in phosphate-buffered saline (PBS) with constant stirring. Aliquots were taken after 5, 10, 20, 30 and 40 min after the reaction. Ten mL of 1% HSA solution was used as an unconjugated control HSA. Ammonium carbonate (2 mol/L) was added to each aliquot to terminate reactions. All reactive samples were centrifuged at 3,000 *g* at 4℃ for 40 min to remove unreactive TDI, extensively dialyzed for 3 days against 0.1 mol/L ammonium carbonate, precipitated with equal volume of 20% trichloroacetic acid, redissolved in 1 mol/L sodium hydroxide, and then dialyzed with 4 L of deionized water for 1 day. When the degree of substitution was determined by modified Gutmann assay, the molar ratio was 5. Protein content of a conjugated sample was determined by Lowry method. All reagents for preparation of TDI-HSA conjugates were purchased from Sigma Chemical Co., U.S.A. Two kinds of TDI-HSA conjugates were donated by Dr. WE Brown at Carnegie Mellon University, Pittsburgh, U.S.A. They were prepared according to the method described in the previous report (12).

Specific IgE-ELISA and ELISA inhibition test

Specific IgE level was detected by ELISA as described previously (8). Diluted patient's serum or negative control serum (50 μL) was added to each well of ELISA plates (Costar Co.) that had been coated with the three kinds of 1 μL/well TDI-HSA conjugates and control HSA dissolved in coating buffer (0.1 mol/L sodium bicarbonate buffer, pH 9.5) under the same experimental conditions, and blocked with 350 μL of blocking buffer (PBS containing 5% BSA and 0.1% Tween 20). After overnight incubation at 37℃, the plates were washed 3 times with phosphate buffered saline tween (PBST). Biotinylated anti-human IgE (50 μL, Sigma Co.) diluted to 1:500 vol/vol with 5% BSA-PBST was incubated for 3 hr at 37℃ and washed with PBST, and then streptavidin-peroxidase (Sigma Co., St. Louis, MO, U.S.A.) was added as substrate. Reactions were stopped with the addition of 2.5N H_2SO_4. The optical density of the solution was determined at 450 nm by using an ELISA reader (Molecular Devices Co., CA, U.S.A.). The final absorbance value of each reaction was calculated by subtraction of the control HSA-coated value from that of the TDI-HSA-coated

Table 1. Comparison of specific IgE bindings to three different TDI-HSA conjugates in 20 sera of TDI-induced asthma

	98 CM conjugate*		99 CM conjugate*	
	Positive	Negative	Positive	Negative
Ajou conjugate				
Positive	3	1	4	0
Negative	0	16	1	15
Positive predictive value	75%		100%	
Negative predictive value	100%		93.8%	

Positive predictive value between two CM conjugates: 100%
Negative predictive value between two CM conjugates: 88.3%
Significant associations were noted between Ajou and 98 CM conjugate (*p=0.004), Ajou and 99 CM conjugate (**p=0.001) and two CM's conjugates (***p=0.009)

value. The positive cut-off values were determined as mean plus 2-folds of standard deviation (S.D.) of the absorbance values from 20 unexposed healthy controls.

For IgE ELISA inhibition assay, sera were pre-incubated overnight with various concentrations of TDI-HSA conjugates (1-100 μg), Hop Japanese pollen antigen as an unrelated antigen, and an equal volume of PBS as a control. Then, they were subjected to IgE-ELISA as described above. Inhibition percentage was calculated as follows:

100−[absorbance of samples pre-incubated with antigens/absorbance of samples pre-incubated with PBS×100 (%)]

Statistical analysis

Cross-tab analysis was applied by using SPSS version 8.0 software (Chicago, MI, U.S.A.) to compare the prevalences of specific IgE between the three different conjugates. A p value less than 0.05 was regarded as significant.

RESULTS

Comparison of specific IgE bindings to three TDI-HSA conjugates by ELISA

Table 1 shows the comparison of specific IgE bindings to three different TDI-HSA conjugates in the same sera. When the results for 98 CM and Ajou conjugates were compared, three had concurrent positive results to both conjugates and 16 had negative results to both conjugates. One showed an isolated positive result to Ajou conjugate with negative results to 98 and 99 CM conjugates. When specific IgE bindings were compared between 99 CM and Ajou conjugates, four had concurrent positive results to both conjugates and 15 had negative results to both conjugates. One patient showed an isolated positive result to 99 CM conjugate with negative results to Ajou and 98 CM conjugates. Positive and negative predictive values between Ajou and 98 CM conjugates were 75% and 100%, respectively. Those between

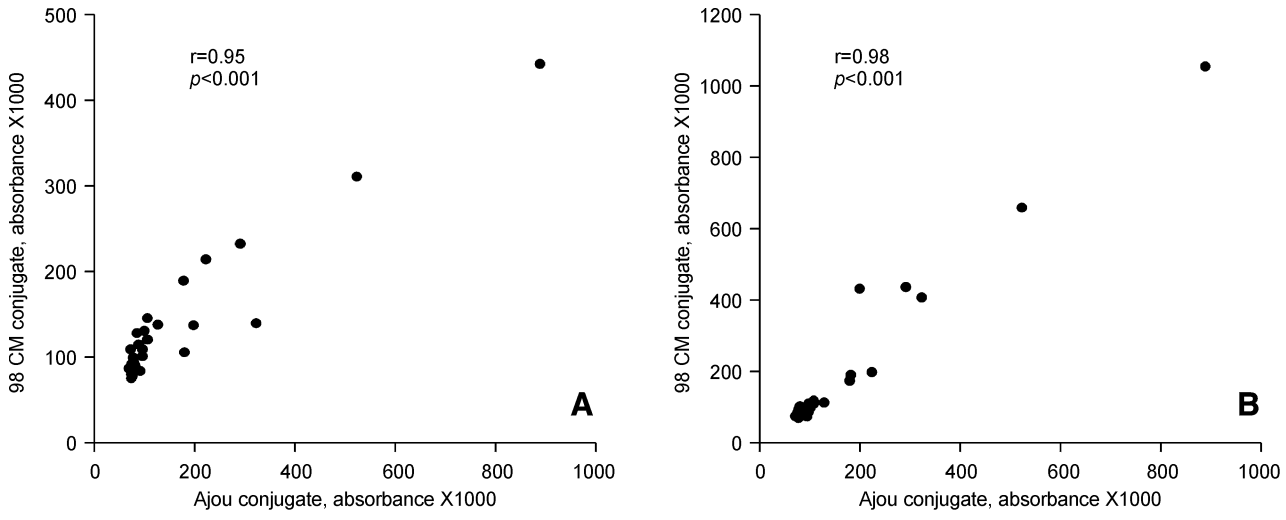

Fig. 1. Correlation between specific IgE bindings to two kinds of conjugates, Ajou and 98 CM (A) and Ajou and 99 CM (B).

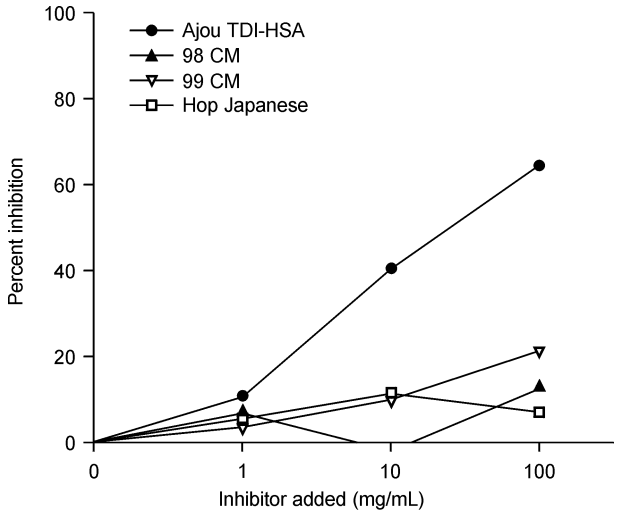

Fig. 2. Percent inhibition of specific IgE bindings to Ajou toluene diisocyanate (TDI)-human serum albumin (HSA) conjugate by ELISA with serial additions of Hop Japanese pollen, three kinds of TDI-HSA conjugates, Ajou and two CM conjugates (98, 99).

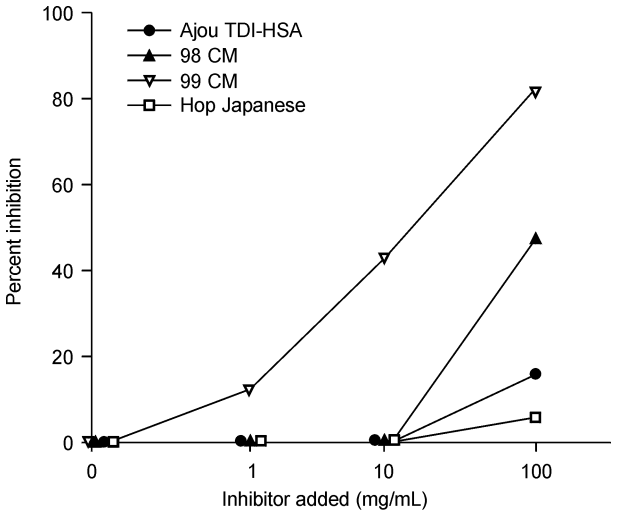

Fig. 3. Percent inhibition of specific IgE bindings to 99 CM toluene diisocyanate (TDI)-human serum albumin (HSA) conjugate by ELISA with serial additions of Hop Japanese pollen, three kinds of TDI-HSA conjugates, Ajou and two CM conjugates (98, 99).

Ajou and 99 CM conjugates were 100% and 93.8%. There were significant correlations in specific IgE levels between Ajou conjugate and 98 CM conjugate ($r=0.95$, $p<0.001$), and between Ajou conjugate and 99 CM conjugate ($r=0.98$, $p<0.001$) as shown in Fig. 1.

Specificity of specific IgE bindings to TDI-HSA conjugate

Fig. 2 shows ELISA inhibition test results using the serum of a subject that had an isolated positive result to Ajou conjugate on IgE-ELISA. Significant inhibitions were noted with additions of Ajou conjugate in a dose-dependent manner, but minimal inhibitions were noted with 98 and 99 CM conjugates, and with Hop Japanese pollen antigen. Fig. 3 shows ELISA inhibition results using the serum of a subject that had an isolated positive result to 99 CM conjugate on IgE-ELISA. Significant inhibitions were noted with additions of 99 CM conjugate and partial inhibition with 98 CM conjugate. Minimal inhibitions were noted with Ajou conjugate and Hop Japanese pollen antigens.

DISCUSSION

Detection of specific IgE in patients with TDI-induced asthma has suggested that IgE-mediated immune response may play a role in the pathogenesis of this chemical allergen-induced asthma. However, investigators have been puzzled by the variable results on the prevalence of specific IgE antibody to TDI-HSA conjugate in TDI-asthma patients. We hypothesized that one of the possible explanations for the failure to detect specific IgE could be heterogeneity of IgE response to TDI-HSA conjugates. The purpose of the present investigation was to compare IgE bindings to three different TDI-HSA conjugates, which were known as the most sensitive ones among the many conjugate pools from each laboratory, and to evaluate individual differences. The individual specificity was confirmed by ELISA inhibition using three kinds of TDI-HSA conjugates.

Individual differences in specificity of IgE antibody responses against various reactive chemicals were reported in previous reports (13, 14). In our study, positive and negative predictive values were high and most positive reactors showed concurrent positive results to the other two conjugates. Moreover, there was a significantly close correlation in specific IgE level among the different conjugates. However, there was one TDI-induced asthma patient having the highest affinity to only the Ajou TDI-HSA conjugate, with no bindings to the other two conjugates. ELISA inhibition test using three kinds of TDI-HSA conjugates showed that significant inhibitions were noted with only the Ajou conjugate in a dose-dependent manner, while minimal inhibitions were noted with the other two conjugates. Moreover, another patient had positive specific IgE binding to 99 CM TDI-HSA conjugate without binding to Ajou or 98 CM conjugate. ELISA inhibition test using this patient's serum showed significant inhibitions with 99 CM conjugate, and minimal inhibitions with the other two conjugates. These findings confirmed the specificity of IgE bindings to the

specific antigenic determinant of TDI-HSA conjugate.

Wass and Belin (9) suggested that the immune response induced by isocyanate could involve both hapten and new antigenic determinants of the carrier protein. The importance of the carrier protein used had been noted by Baur (10) who found a variable IgE response to TDI by RAST according to carrier proteins used. Electrically charged hydrophilic groups of carrier protein such as $-NH_3$, on the surface of a macromolecule may be the preferential site reacting with isocyanate. Although precise antigenic determinants of each patient's specific IgE antibody remain to be further investigated, there is a possibility that TDI-HSA may undergo particular changes in three-dimensional configuration, resulting in generation of new antigenic epitopes. Antibodies against new antigenic determinants on the TDI-HSA could be produced in vivo. Therefore, the immune response to TDI-carrier conjugates generated in vivo may vary among individuals sensitized to TDI. These differences may be one of the possible explanations for individual differences in IgE response patterns, which were observed in our experimental results.

In conclusion, individual differences and their specificities in specific IgE bindings to a new antigenic determinant of TDI-HSA conjugates were confirmed in this study, which may be responsible for the variable results in the prevalence of serum specific IgE antibody to TDI-HSA conjugate in TDI-induced asthma.

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