J Korean Med Sci 1998; 13: 147-52
ISSN 1011-8934

Heterogeneity of IgE Response to TDI-HSA Conjugates by ELISA in Toluene Diisocyanate (TDI) -induced Occupational Asthma (OA) Patients

Toluene diisocyanate (TDI), a low molecular weight reactive chemical, is known to be a main cause of occupational asthma (OA) in Korea. Although it is thought that inhaled TDI may act as a hapten, the precise mechanisms of TDI-induced OA are unknown. In this study, TDI-human serum albumin (HSA) conjugates (5, 10, 20 and 30 min) were prepared in the range of 1.5 to 5.0 TDI mole/HSA mole. Specific binding of serum IgE to TDI-HSA (30 min) was observed using IgE ELISA as well as ELISA inhibition assay. Around 40% of TDI-induced OA patients were positive for serum specific IgE by ELISA. Degrees of serum IgE binding were different depending on which TDI-HSA conjugate was used as an antigen. Moreover, binding patterns were different depending on the individuals. Interestingly, higher binding of IgE to TDI-HSA (5 min) than to TDI-HSA (30 min) which was more highly substituted was observed in some patients. Probably new antigenic epitopes on carrier proteins were targets of the specific IgE. The results of this study indicated that IgE responses to TDI-HSA conjugates were heterogenous in TDI-induced OA patients and self-proteins modified by reactive chemicals can become a major target antigen of IgE in certain cases.

Key Words : *IgE; Toluene 2,4-diisocyanate; Haptens; Asthma, Occupational diseases*

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Received : September 23, 1997
Accepted : January 5, 1998

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* This work was supported by a grant from the Han Dok Pharmaceutical Company.

INTRODUCTION

Toluene diisocyanate (TDI) is a highly reactive chemical which is extensively used for manufacturing polyurethane foam, adhesives, paints, lacquers, and many other products. At present, TDI is regarded as the most prevalent cause of occupational asthma (1). Although involvement of the immune mechanism in the pathogenesis of TDI-induced occupational asthma (OA) has been suggested (1), specific IgE antibodies have been documented in only 15 to 20% of affected workers using radioallergosorbent tests (RAST) (2-5). Due to the difficulties in detecting serum specific IgE, a pathogenic role for IgE-mediated immune response has been controversial.

We have questioned why some patients become IgE-sensitized to TDI-human serum albumin (HSA), while no specific IgE is detected in others. The possibility could not be excluded that the RAST that we have used was not sensitive enough to detect heterogeneous IgE antibodies against various antigenic determinants on the TDI-HSA conjugates. This study was initiated to determine whether there are any differences in binding of specific IgE depending on different preparations of TDI-HSA conjugates. Using IgE ELISA, we could observe individual differences in specificities of IgE.

MATERIALS AND METHODS

Patients and reagents

Sera were obtained from patients diagnosed as having TDI-induced occupational asthma proven by TDI-bronchial challenge test (6). All reagents and antibodies used for preparation of TDI-HSA conjugates and IgE ELISA were purchased from Sigma Chemical Co. except sodium 2-naphthol-3,6-disulfate which was purchased from Acros Organics (Belgium).

Preparation of TDI-HSA conjugates

TDI-HSA conjugates were prepared by a modification of Tse and Pesce's method (7). Briefly, 2.4 g of TDI (toluene-2,4-diisocyanate) was added to 90 ml of 1%

HSA in phosphate buffered saline with constant stirring, and 10 ml of aliquot was taken at 5, 10, 20 and 30 min after the beginning of the reaction. Ten ml of 1% HSA solution was used as an unconjugated control-HSA. Ammonium carbonate (2 M) was added to each aliquot to terminate reactions. All reacted samples including the control HSA were centrifuged at 3,000 *g* for 20 min at room temperature to remove unreacted TDI, dialyzed (Cellulose membrane, molecular weight cut off 12,000, D9777, Sigma Chemical Co.) for 3 days with 4 liters of 0.1 M ammonium carbonate, precipitated with equal volume of 20% trichloroacetic acid, redissolved in 1 M sodium hydroxide, and then dialyzed overnight three times with 4 liters of distilled water.

Determination of amount of TDI bound to HSA

Degrees of substitution were determined by a modified Gutmann assay (8). The assay was performed on 50 μl of aliquots of samples to which were added 50 μl of concentrated HCl, 50 μl of D.W. and 0.2 ml of 0.029 M sodium nitrate. After 5 min of reaction, 0.6 ml of 0.031 M sodium 2-naphthol-3,6-disulfonate in 7.4 M ammonium hydroxide was added. Each assay sample was mixed thoroughly, allowed to stand for 5 min, and read spectrophotometically at 500 nm. The amount of isocyanate bound to HSA was measured using a standard calibration curve constructed from different concentrations of *p*-toluidine. The protein content of a conjugated sample was determined by Lowry method (9) using bovine serum albumin (BSA) as a standard.

Polyacrylamide gel electrophoresis (PAGE)

Conjugates were electrophoresed through 8% polyacrylamide gel together with molecular weight standards (BSA (monomer); 66 kDa, BSA (dimer); 132 kDa, urease (trimer); 272 kDa, urease (hexamer); 545 kDa). The gel was stained with 0.1% Coomassie Brilliant Blue R-250.

IgE ELISA and IgE ELISA inhibition assay

Patients' sera or negative control sera were added to each well of ELISA plates (#3590, Costar, Cambridge, MA), which had been coated overnight with 10 μg of TDI-HSA conjugate or control HSA dissolved in 200 μl of coating buffer (0.1 M sodium bicarbonate buffer, pH 9.5) and blocked with blocking buffer (PBS containing 5% BSA, 0.1% Tween 20 and 0.2% sodium azide) for 1 hr. After overnight incubation in the presence of patients sera at 37℃, plates were washed three times with PBST (PBS containing 0.1% Tween 20 and 0.2% sodium azide). Biotinylated anti-human IgE (Sigma Chemical Co., BA-3040) diluted to 1:1000 with 5% BSA-PBST was added into each well. Plates were incubated for 3 hr at 37℃, washed with PBST, and then alkaline phosphatase conjugated streptavidin (Sigma Chemical Co., S2890) diluted to 1:8000 with 5% BSA-PBST was added into each well. After incubation for 1hr at 37℃, plates were washed and substrate solution (*p*-nitrophenyl phosphate) was added. Optical density at 405 nm (O.D. at 405 nm) was determined using an ELISA reader (Molecular Devices Co., USA).

For IgE ELISA inhibition assay, sera were preincubated with various concentrations of TDI-HSA conjugates (1 pg to 1 mg) overnight, and then subjected to IgE ELISA as described above.

RESULTS

Preparation of TDI-HSA conjugates

Various TDI-HSA conjugates were prepared at different time points of reaction. As Fig. 1 shows, the amount of TDI bound to HSA was found to increase as the reaction time increased up to 30 min, at which time the degree of substitution was maximal. Under conditions that we used in this experiment, only a minimal amount of protein was recovered from samples reacted longer

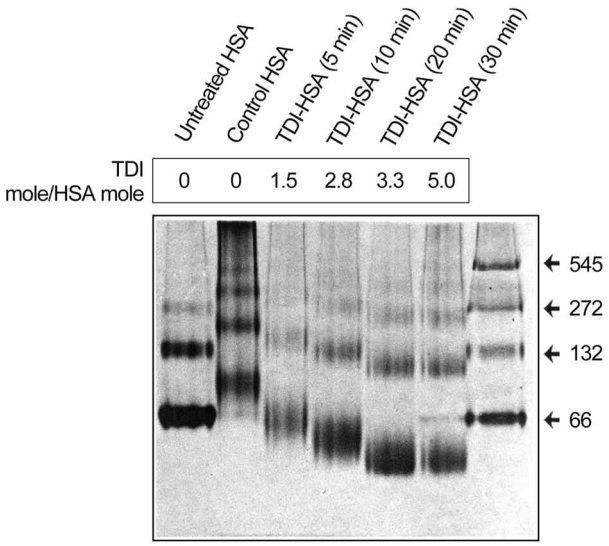

Fig. 1. Preparation of TDI-HSA conjugates. TDI was added to 1% HSA and aliquots were taken at various reaction time. The amount of isocyanate bound to each conjugate sample was determined by Gutmann assay and Lowry protein assay as described in Materials and Methods. Conjugates were analyzed using 8% non-denatured PAGE. TDI-HSA (5 min), TDI-HSA (10 min), TDI-HSA (20 min) and TDI-HSA (30 min) represent TDI-HSA conjugates reacted for 5, 10, 20 and 30 min respectively.

than 40 min. To ensure successful preparations of TDI-HSA conjugates, samples were analysed using non-denatured PAGE. Changes in mobility of HSA were observed following conjugation with TDI. The mobility of the conjugates was found to increase as the amount of bound TDI increased (Fig. 1). Four different TDI-HSA conjugates with various substitutions, from 1.5 mol TDI/mol HSA to 5 mol TDI/mol HSA, were used for further experiments.

Reaction of various preparations of TDI-HSA conjugates with specific IgE

To investigate whether the prepared conjugates are specifically reacted with serum IgE antibody, IgE ELISA was performed using a known positive serum from a TDI-induced OA patient, which had been tested by RAST (11). To ensure specific binding of serum IgE to TDI-HSA (30 min), variously diluted positive serum was subjected to IgE ELISA using various concentrations of TDI-HSA (30 min). As Fig. 2 shows, binding of IgE to TDI-HSA (30 min) declined as the concentration of the antigen decreased. Similar findings were observed when both 1:10 and 1:100 serum were used, and IgE binding was higher when serum with lower dilution was used. Specificity of IgE binding to the conjugate was further confirmed by ELISA inhibition assay. Preincubation of the positive serum with TDI-HSA (30 min) inhibited binding of IgE to the antigen in a dose-dependent manner (Fig. 3).

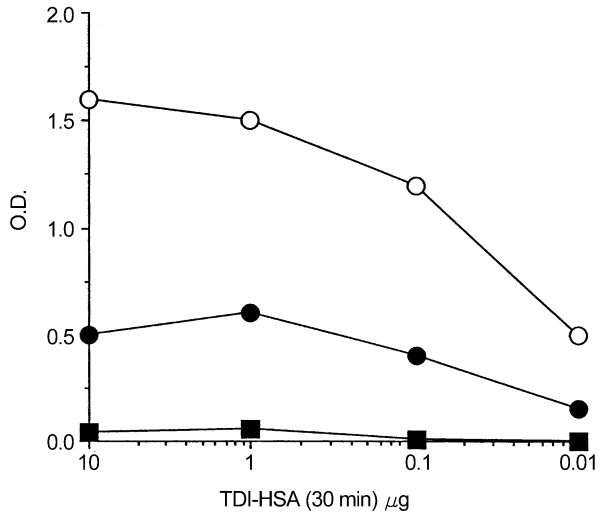

Fig. 2. Specific binding of IgE to TDI-HSA (30 min). Reactions of various concentrations of TDI-HSA (30 min) with a known positive serum with various dilutions were determined by IgE ELISA (-○-, 1:10; -●-, 1:100; -■-, 1:1000).

Individual differences in specificity of IgE

To investigate whether there are any differences in specificity of IgE, two different conjugates, TDI-HSA (5 min) and TDI-HSA (30 min), were reacted with sera from three recently diagnosed TDI-induced OA patients. Distinct patterns of IgE binding to the conjugates were observed among different individuals (Fig. 4).

Patient A showed higher IgE binding to TDI-HSA (30 min) than TDI-HSA (5 min). In contrast, patient C showed higher IgE binding to TDI-HSA (5 min) than TDI-HSA (30 min). Meanwhile, patient B showed no significant difference in IgE binding between two conjugates (Fig. 4).

Changes in titers of specific IgE

To investigate changes of specific IgE in the sera of patients who avoided exposure to TDI after the diagnosis, four different preparations of TDI-HSA conjugates were reacted with patients' sera. Three out of seven patients showed positive for specific IgE. Different patterns of IgE response to the TDI-HSA conjugates were observed among the sera of initial diagnosis (Fig. 5). In patient L, lower titer of specific IgE was detected 1 month after the diagnosis, while no specific IgE was detected in the other two patients in which follow-up sera were obtained 1 yr and 6 yr after the diagnosis (Fig. 5). In this particular patient, patterns of IgE response

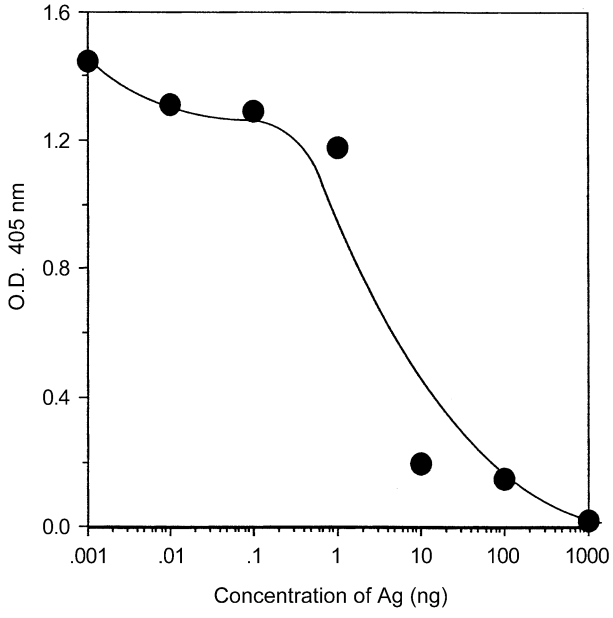

Fig. 3. Results of ELISA inhibition assay. The positive sera were preincubated with various concentrations of TDI-HSA (30 min) (1 pg to 1 mg) and then subjected to IgE ELISA as described in Materials and Methods.

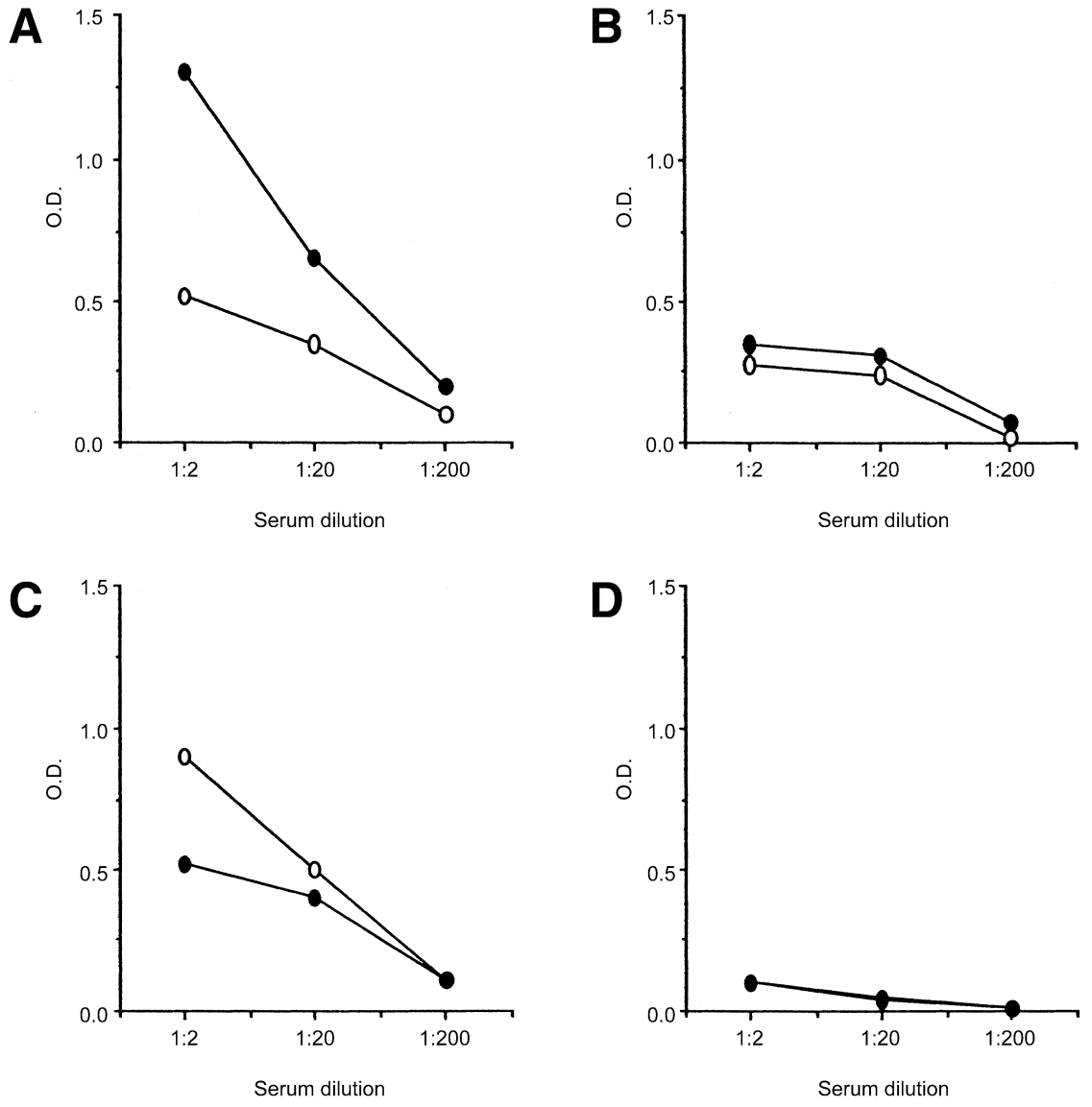

Fig. 4. Individual differences in specificity of IgE antibody. IgE bindings to two different TDI-HSA conjugates (-○-, TDI-HSA (5 min); -●-, TDI-HSA (30 min)) were examined by IgE ELISA. Panel A, B, C, TDI-induced OA patients; Panel D, normal healthy control.

were not consistent. While the highest IgE binding to TDI-HSA (30 min) was shown at the time of diagnosis, the highest IgE binding to TDI-HSA (5 min) was shown one month later (Fig. 5).

DISCUSSION

Detection of specific IgE in patients with TDI-induced OA 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 low prevalence of specific IgE among TDI-induced asthma patients (2-5, 10, 11). We hypothesized that one of the possible explanations for the failure to detect specific IgE could be improper preparations of TDI-HSA conjugates. The purpose of the present investigation was to determine whether specificity of serum IgE can differ depending on the preparations of TDI-HSA conjugates or depending on the individual variations. To answer this question, various TDI-HSA conjugates were prepared under conditions of different reaction times and the conjugates were characterized by degrees of substitution with isocyanates and also by changes in mobility on the gel.

Studies on antigenic determinants on TDI-HSA con-

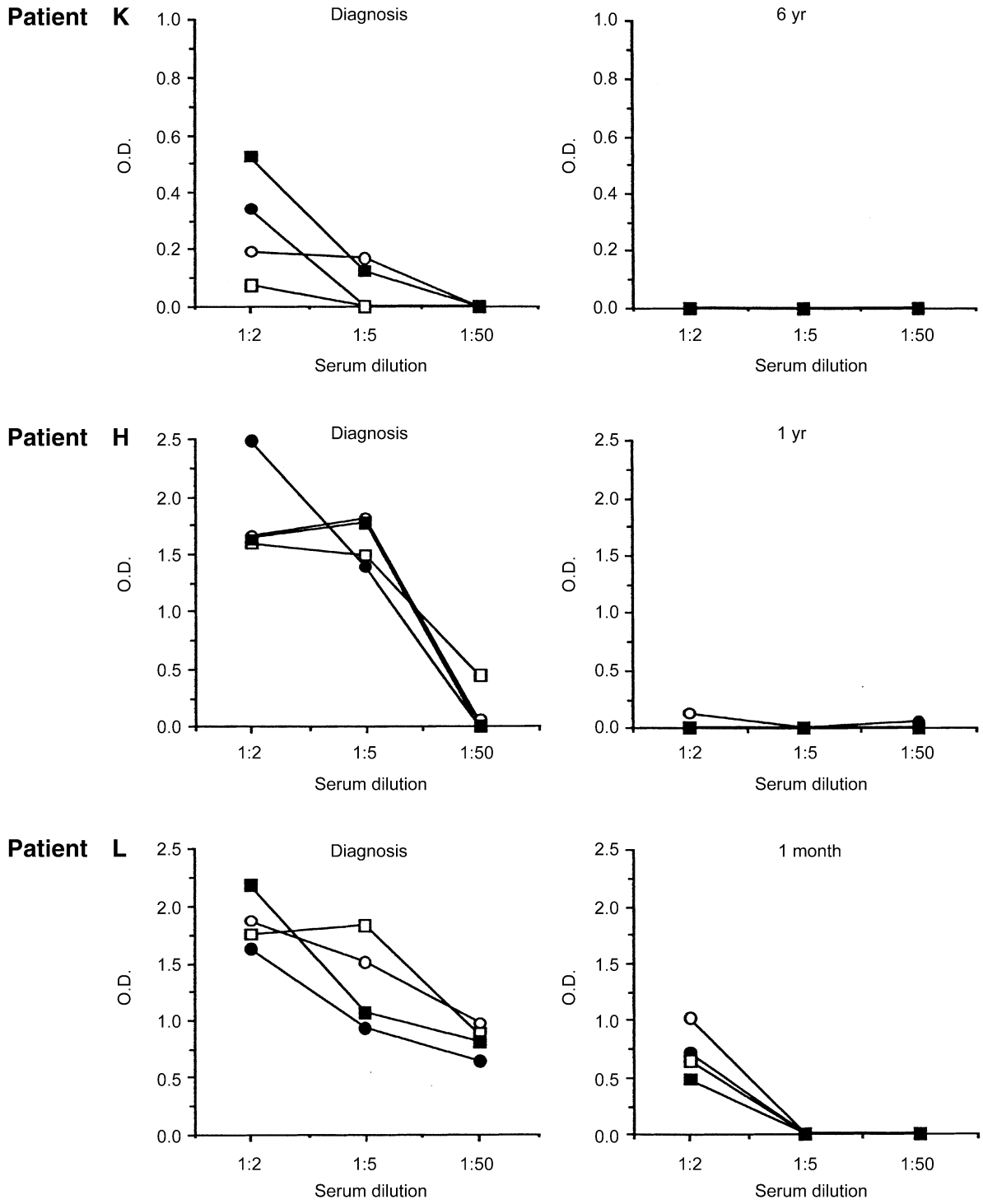

Fig. 5. Changes in titers of specific IgE. IgE bindings to four different TDI-HSA conjugates (-○-, TDI-HSA (5 min); -●-, TDI-HSA (10 min); -□-, TDI-HSA (20 min); -■-, TDI-HSA (30 min)) were examined by IgE ELISA. Paired sera from patients were obtained at the time of initial diagnosis and clinical follow-up.

jugates, to which specific IgE binds, have indicated that heterogeneous populations of IgE may be present in the patients' sera (12, 13). Specific IgE may develop against TDI itself, or against linkage site between TDI and HSA, or against neoantigens 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 conju-

gates at lower degree of substitution were found to bind specific IgE better than TDI-HSA conjugates highly substituted in RAST assays (14).

Individual differences in specificity of IgE antibody response against various TDI-HSA conjugates were observed in the present study. Interestingly, while some TDI-induced OA patient's sera were found to have highest affinity to a TDI-HSA conjugate (30 min), some patient's sera IgE were found to have higher affinity to TDI-HSA (5 min) than TDI-HSA (30 min) which was more highly substituted. Although precise antigenic determinants of this patient's specific IgE remain to be further investigated, there is a possibility that TDI-HSA with high substitution may have particular changes of three-dimensional configuration of the carrier protein, resulting in generations of new antigenic epitopes. Charged hydrophilic groups on the surface of the carrier protein may be the preferential sites reacting with an isocyanate of TDI. This interaction may lead to a change of the three-dimensional configuration of the carrier protein (i.e. HSA). Therefore, antibodies against new antigenic determinants on the HSA could be produced in vivo. It is likely that the immunogenicity of a TDI-HSA conjugate may vary depending on the degree of conjugation. Presumably, a conjugate with low substitution would be sterically intact and induce mainly hapten-specific antibodies. In contrast, a conjugate heavily substituted would lead to strong immunogenicity for the carrier protein. It can be expected that hapten-carrier conjugates generated in vivo may be various among individuals sensitized to TDI. These differences in the in vivo generation of conjugates may be one of the possible explanations for individual differences in IgE response patterns which were observed in our experimental results.

In this study, we found TDI-HSA-specific serum IgE in no more than 30% of patients with suspected isocyanate-induced respiratory symptoms, even though various conjugates were used as antigens. Furthermore, follow-up studies showed that serum specific IgE did not last for a long time. Therefore it was still not easy to detect specific IgE in the sera of TDI-induced occupational asthma patients. The results, however, may suggest that specific IgE may play a role, if any, in the initiation of TDI-induced asthma. Further studies may provide a clue to understanding allergic reactions to low-molecular weight chemical haptens.

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