

Serum specific IgG response to toluene diisocyanate-tissue transglutaminase conjugate in toluene diisocyanate-induced occupational asthmatics

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ABSTRACT

Background: Tissue transglutaminase (tTG) is a post-translational modifying enzyme located in airway epithelial cells. A potential contribution of serum specific IgG (sIgG) to tTG in airway inflammation of toluene diisocyanate (TDI)-induced occupational asthma (OA) has been suggested.

Objective: To prepare a TDI-tTG conjugate and detect serum specific antibodies in sera of patients with TDI-OA to understand this mechanism.

Methods: Ninety-nine patients with TDI-OA, 76 asymptomatic exposed controls, 208 patients with non-OA, and 74 unexposed controls were enrolled for this study. The TDI-tTG conjugate was prepared and confirmed by a native gel. Serum sIgG and/or sIgE antibodies to tTG, TDI-tTG, TDI conjugated to human serum albumin, cytokeratin 19, and serum cytokine levels, such as interleukin-8, transforming growth factor- β 1, and tissue inhibitor of metalloproteinase-1, were measured by enzyme-linked immunosorbent assay. The level of interleukin-8 produced from airway epithelial cells (A549) treated with tTG was evaluated to investigate the inflammatory effect of tTG and TDI-tTG.

Results: In the TDI-OA group, the prevalence of serum sIgG to TDI-tTG (17.2%) was higher than that of sIgG to tTG (11.1%), which were significantly higher than those of the 3 control groups ($P < .05$ for all groups). TDI-exposed subjects with high levels of serum sIgG to TDI-tTG had a high prevalence of sIgG to cytokeratin 19 and higher serum levels of transforming growth factor- β 1 and tissue inhibitor of metalloproteinase-1. The tTG and TDI-tTG dose-dependently increased interleukin-8 production from A549 cells.

Conclusion: These findings suggest that TDI exposure in the workplace binds to tTG to form a conjugate that can induce serum sIgG antibody production, airway inflammation, and airway remodeling in patients with TDI-OA.

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Introduction

Toluene diisocyanate (TDI) is a principal cause of occupational asthma (OA).¹ Similarly to other low-molecular-weight agents, TDI binds to tissue proteins to form conjugates that can induce immune responses and elicit the production of specific IgE (sIgE) and specific IgG (sIgG) antibodies to conjugates, such as TDI to human serum albumin conjugate (TDI-HSA).^{1,2} However, the immunologic mechanisms of TDI-induced airway sensitization and inflammation are not completely understood.³

Tissue transglutaminase (tTG), a post-translational protein-modifying enzyme, contributes to the pathologic mechanism of various autoimmune diseases. Tissue TG is located in the cell nucleus, the cytosol, on cell surfaces, and in the extracellular matrix^{4–6} and is found in various cell types, such as endothelial cells, smooth muscle cells, fibroblasts, macrophages, and airway epithelial cells.^{7–9} Tissue TG has been proposed to relate to the loss of tolerance and induction of autoimmune diseases by the generation of new epitopes by crosslinking with exogenous antigens.⁸ The authors previously reported that TDI can activate tTG in human airway epithelial cells (HAECs) through reactive oxygen species (ROS) generation; in addition, the prevalence of serum sIgG antibody to tTG in patients with TDI-OA was significantly higher than in TDI-exposed and unexposed controls.¹⁰

Cytokeratin (CK) is a major protein of airway epithelial cells. The authors previously reported that TDI exposure can augment CK19

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expression and elicit the production of sIgG autoantibody to CK19 in sera of patients with TDI-OA.¹¹ In addition, tTG has been reported to crosslink CK19, which may influence the development of antibodies to CK19.¹⁰ In consequence, a crosstalk among TDI, tTG, and CK19 that contributes to the immunologic mechanism of TDI-OA has been suggested by previous studies.

In the present study, the authors hypothesized that TDI exposure could bind to tTG located in HAECs to induce immune and airway inflammatory responses. Therefore, they prepared a TDI-tTG conjugate and detected serum specific antibodies to TDI-tTG in TDI-exposed subjects compared with unexposed controls. They also evaluated the association between specific antibodies to tTG and TDI-tTG with sIgG to CK19 and levels of various serum cytokines related to airway inflammation and remodeling.¹² The inflammatory effects of tTG and TDI-tTG on interleukin (IL)-8 production from HAECs were investigated by in vitro experiments. Existing immunologic tests (such as sIgG or sIgE to TDI-HSA conjugate) are not sensitive enough to serve as diagnostic tests for TDI-OA; therefore, they evaluated the diagnostic values of sIgG to tTG and TDI-tTG to determine whether these values would provide better serologic tests for the diagnosis of TDI-OA.

Methods

Subject Recruitment

This study enrolled 457 study subjects. TDI-exposed subjects included 99 patients with TDI-OA and 76 asymptomatic exposed controls (AECs). Study subjects were evaluated by a questionnaire survey of TDI workplace exposure and work-related asthmatic symptoms. Sera from all subjects were collected at initial evaluation. TDI-OA was diagnosed based on positive results to methacholine and TDI-specific bronchial challenge tests. Patients with TDI-OA used inhaled corticosteroids with or without a long-acting β_2 agonist (but not an oral corticosteroid) as maintenance medication. TDI-unexposed control groups included 208 adult patients with non-OA and 74 healthy normal controls (NCs). Atopy status was defined as at least 1 positive reaction to skin prick tests using the following common aeroallergens (Bencard, Bradford, United Kingdom): *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cat, dog, cockroach, tree pollen mixture (including alder, ash, beech, birch, elm, hazel, oak, plane, poplar, willow), grass pollen mixture (including velvet grass, orchard, rye grass, timothy grass, Kentucky blue grass, meadow fescue), mugwort, ragweed, Japanese hops, aspergillus, and *Alternaria* species. All participants provided written informed consent as regulated by the institutional review board of Ajou University Hospital, Suwon, South Korea (AJIRB-05-200).

Methacholine and TDI-Specific Bronchial Challenge Tests

Methacholine bronchial challenge tests were performed as previously described with doubled doses of methacholine (0.075 to 25 mg/mL).¹³ Interpolation from a dose–response curve determined the concentration of methacholine needed to produce a 20% decrease in forced expiratory volume in 1 second. TDI bronchial challenge tests were conducted within 1 month after the last TDI exposure, as described previously.¹³

TDI-tTG Conjugate Production

Human recombinant tTG produced and characterized as previously described¹⁴ was kindly provided by Professor Chaitan Khosla (Stanford University, Stanford, California). The 2,4-TDI was dissolved in 1,2-dimethoxyethane (Sigma, St Louis, Missouri) as a solvent to obtain the TDI solution (5 mol/L). Then, 20 μ L of TDI solution (5 mol/L) was added to 980 μ L of a tTG solution (80 μ g/mL, diluted in phosphate buffer saline [PBS]) to produce TDI-tTG;

subsequently, the mixture was mixed by vortex and incubated for 3 minutes at room temperature (RT) to obtain the TDI-tTG conjugate solution.

Native Polyacrylamide Gel Electrophoresis

Tissue TG or TDI-tTG were electrophoresed on 10% native polyacrylamide gel and stained with 0.1% Coomassie Brilliant Blue G-250 (Amresco, Solon, Ohio).

Enzyme-Linked Immunosorbent Assay to Detect Serum sIgG Antibodies to tTG and TDI-tTG

In the preliminary experiments to optimize enzyme-linked immunosorbent assay (ELISA) conditions, 10 serum samples from the TDI-OA, AEC, asthma, and NC groups were selected randomly. Each sample was tested with a set of antigen-coated and noncoated wells. Different concentrations of coating antigens (80 to 8,000 ng/mL of tTG and TDI-tTG) for each dilution ratio of human serum samples (1:10 to 1:1,000) were tested. Blanks were conducted by adding blocking buffer to antigen-coated wells as negative controls. Different incubation times were tested for each condition. The optical density values of all noncoated wells did not differ from those of the blanks and were much lower than those of coated wells (data not shown). Based on the preliminary results, the optimal condition that provided good optical densities with the lowest background was selected for further experiments.

To prepare coating antigens, tTG (80 μ g/mL) and TDI-tTG (80 μ g/mL) solutions were separately diluted in 0.05 mol/L of NaHCO₃ buffer to obtain 8- μ g/mL coating mixtures. Then, 100 μ L of each mixture was coated onto each well of 96-well ELISA microplates (Corning Incorporated, Corning, New York) and incubated at 4°C overnight. After washing 3 times with PBS that contained 0.05% Tween 20 (PBS-T), the plates were blocked with 200 μ L of blocking buffer (PBS-T that contained 3% skim milk; Difco, Detroit, Michigan) for 1 hour at RT. The plates were washed again 3 times, and the diluted serum (1:500 in blocking buffer, 100 μ L) from all subjects was added to the tTG- and TDI-tTG-coated wells and incubated for 3 hours at RT. After washing 3 times, 100 μ L of alkaline phosphatase (AP)-conjugated antihuman IgG antibody (1:250; Sigma) was added to each well and incubated for 3 hours at RT. After washing 5 times, 100 μ L of *p*-nitrophenyl phosphate substrate (Sigma) was added to each well and incubated for 20 minutes at RT. The reactions were stopped by 100 μ L of NaOH (1 N). Optical densities were measured at a 405-nm wavelength on a microplate reader (Synergy HT; BioTek Instrument, Inc, Winooski, Vermont). The positive cutoff value for each antigen was determined by the mean plus 3-fold SD of absorbance values from tests in healthy controls.

ELISA Inhibition Test

Binding specificities of sIgG to tTG and TDI-tTG were evaluated by ELISA inhibition tests. Increasing amounts (1 to 40 μ g/mL) of tTG and TDI-tTG were incubated overnight with 100- μ L diluted (1:500) serum samples from 3 different subjects: 1 had a high level of sIgG to TDI-tTG (but not to tTG); 1 had a high level of sIgG to tTG (but not to TDI-tTG); and 1 had high levels of sIgG to tTG and the TDI-tTG conjugate. The mixtures were applied to the ELISA as described earlier. The percentage of inhibition was calculated as $100 \times (1 - [\text{absorbance with inhibitor}/\text{absorbance without inhibitor}])$.

ELISA to Detect Serum sIgG and sIgE Antibodies to TDI-HSA Conjugate

Serum sIgG and sIgE antibodies to TDI-HSA conjugate were detected using ELISA as previously described.¹⁵ Vapor-type TDI-HSA conjugate was kindly provided by Dr Adam Wisniewski (Yale University, New Haven, Connecticut). First, 1 μ g of TDI-HSA or mock

conjugate was dissolved in normal saline and used to coat the ELISA microplates. After washing with PBS-T, plates were blocked with a blocking buffer. Because skim milk yielded high backgrounds in this ELISA setting, a blocking buffer with PBS-T containing 5% bovine serum albumin was used instead. Serum samples from study subjects were diluted 1:5 for IgG and 1:2 for IgE in blocking buffer. Then, 50 μ L of each diluted serum sample was added and incubated for 1 hour at RT in the TDI-HSA and mock-HSA coated wells. After washing 4 times, AP-conjugated anti-IgG antibody (1:2,000, 100 μ L; Sigma) or biotinylated anti-IgE antibody (1:1,000; Kirkegaard & Perry Laboratories, Gaithersburg, Maryland) followed by AP-conjugated streptavidin (1:500; Sigma) was added to each well and incubated for 1 hour at RT. The plates were washed and 100 μ L of a substrate solution (Sigma) was added. After incubating for 15 minutes at RT, absorbance values were read using an ELISA reader at 405 nm and 450 nm. To determine the antibody binding specific to TDI, the absorbance of TDI-HSA-coated wells was subtracted from the absorbance of the mock-HSA-coated wells. Positive cutoff values were calculated by the mean plus 3-fold SD of optical density values from tests in healthy controls.

ELISA to Detect sIgG Antibody to CK19

Specific IgG antibody to CK19 was detected using ELISA as previously described.¹⁶ Each well of the ELISA microplates was coated with 100 ng of CK19 (Research Diagnostics, Flanders, New Jersey) dissolved in PBS. After blocking the plates with 200 μ L of PBS containing 10% fetal bovine serum, 50 μ L of diluted (1:5) serum samples from study subjects was added to each well and incubated for 1 hour at RT. The plates were washed 3 times with PBS-T and incubated with 100 μ L of AP-conjugated antihuman IgG (1:10,000; Sigma) for 1 hour at 37°C. *p*-nitrophenyl phosphate substrate (Sigma) was added as a substrate after washing the plates 3 times. NaOH (1 N) was used to stop the reaction in the wells. The optical densities were measured using an ELISA reader at 405 nm. The final absorbance was calculated by subtracting the absorbance of the uncoated wells from that of the coated wells. The positive cutoff values were determined as the mean plus 2-fold SD of absorbance values from tests in unexposed healthy controls.

ELISA to Measure Serum Cytokine Levels

Levels of serum cytokines, including transforming growth factor- β 1 (TGF- β 1), tissue inhibitor of metalloproteinase-1 (TIMP-1), matrix metalloproteinase-9, myeloperoxidase, IL-8, and vascular endothelial growth factor, were measured using commercial ELISA kits (TGF- β 1, metalloproteinase-9, IL-8, and vascular endothelial growth factor: R&D Systems, Inc, Minneapolis, Minnesota; myeloperoxidase: BioCheck, Inc, Foster City, California; TIMP-1: catalog number RPN2618; Amersham Pharmacia Biotech, Amersham, United Kingdom) as previously described.¹⁶

IL-8 Production from HAECs Stimulated with tTG and TDI-tTG Conjugate

Human airway epithelial A549 cells were purchased from American Type Culture Collection (Manassas, Virginia). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, New York) with 10% fetal bovine serum (Gibco) and maintained at 37°C with 5% CO₂ in humidified air. Cells (2×10^5) were seeded in 12-well plates (TPP, Trasadingen, Switzerland) and subsequently treated with different concentrations of tTG (100, 10, 1, 0.1 μ g/mL), TDI-tTG (100, 10, 1, 0.1 μ g/mL), TDI-HSA (200, 100, 50 μ g/mL), or serum-free medium as a control. After 24 hours of incubation, the supernatants were collected for IL-8 measurement by a commercial ELISA kit (Endogen, Woburn, Massachusetts).

Table 1

Clinical and immunologic features of study subjects

	TDI-OA (n = 99)	AEC (n = 76)	Asthma (n = 208)	NC (n = 74)	P value
Age (y) ^a	42.39 $\pm$ 9.86	40.39 $\pm$ 8.5	44.6 $\pm$ 12.8	26.43 $\pm$ 3.4	<.001
Men ^b	68/98 (69.4)	59/76 (77.6)	57/184 (30.9)	50/74 (67.6)	<.001
Exposure period (y) ^a	6.29 $\pm$ 4.26	11.65 $\pm$ 7.20	NA	NA	.001
Asthma duration (y) ^a	7.06 $\pm$ 3.9	NA	6.54 $\pm$ 7.3	NA	NS
Atopy ^{b,c}	36/79 (45.6)	NA	72/142 (50.1)	24/55 (43.6)	NS
Smoking ^b	27/80 (33.8)	35/56 (62.5)	28/92 (30.4)	NA	<.001
FEV ₁ (%) ^{a,c}	84.39 $\pm$ 22.8	90.17 $\pm$ 20.2	83.5 $\pm$ 21.6	99.3 $\pm$ 5.2	NS
PC20 (mg/mL) ^{a,c}	7.85 $\pm$ 14.8	7.9 $\pm$ 9.35	7.9 $\pm$ 15.1	NA	NS
sIgG to TDI-HSA ^{b,d}	22/87 (25.3)	8/76 (10.5)	NA	0/10 (0)	.015
sIgE to TDI-HSA ^{b,c}	21/88 (23.9)	3/76 (3.9)	NA	0/10 (0)	<.001
sIgG to CK19 ^{b,d}	17/97 (17.5)	1/76 (1.3)	NA	0/63 (0)	.001
Total IgE (kIU/L) ^{a,d}	0.318 $\pm$ 0.48	0.308 $\pm$ 0.76	0.388 $\pm$ 0.75	0.114 $\pm$ 0.18	NS

Abbreviations: AEC, asymptomatic exposed controls; CK19, cytokeratin 19; FEV₁, forced expiratory volume in 1 second; NA, not available; NC, unexposed healthy controls; NS, not significant; PC20, concentration of methacholine required to produce a 20% decrease in forced expiratory volume in 1 second; sIgE, serum specific IgE antibody; sIgG, serum specific IgG antibody; TDI-HSA, toluene diisocyanate conjugated to human serum albumin; TDI-OA, patients with toluene diisocyanate-induced occupational asthma.

^aData are presented as mean $\pm$ SD; P values were obtained by 1-way analysis of variance.

^bData are presented as prevalence (percentage); P values were obtained by χ^2 test.

^cData from in vivo tests.

^dData from in vitro experiments.

Statistical Analysis

Demographic data of the 4 study groups were compared using 1-way analysis of variance and χ^2 test. The levels and prevalence of sIgG to tTG and TDI-tTG were compared using the Student *t* test and χ^2 test. The Mann-Whitney *U* test and Fisher exact test were applied to compare the clinical parameters of study subjects according to the responses of sIgG to tTG and TDI-tTG. The IL-8 levels among the treated cell groups were compared using 1-way analysis of variance. All statistical analyses were conducted using SPSS 20.0.0 (SPSS, Chicago, Illinois) and a P value less than .05 was judged significant.

Results

Clinical Characteristics of Study Subjects

Table 1 presents the demographic data of the study subjects. The mean age was significantly younger in the NC group compared with the 3 other groups ($P < .001$). There was a male predominance in the TDI-OA, AEC, and NC groups. The TDI exposure period of the TDI-OA group (6.29 $\pm$ 4.26 years) was significantly shorter than that



Figure 1. Native gel analysis of tissue transglutaminase and conjugate of tissue transglutaminase and toluene diisocyanate. Lane 1, tissue transglutaminase; lanes 2 to 6, conjugate of tissue transglutaminase and toluene diisocyanate produced 3, 5, 10, 20, and 30 minutes before electrophoresis. Arrow indicates tissue transglutaminase-specific band; arrowhead indicates toluene diisocyanate and tissue transglutaminase-specific band.

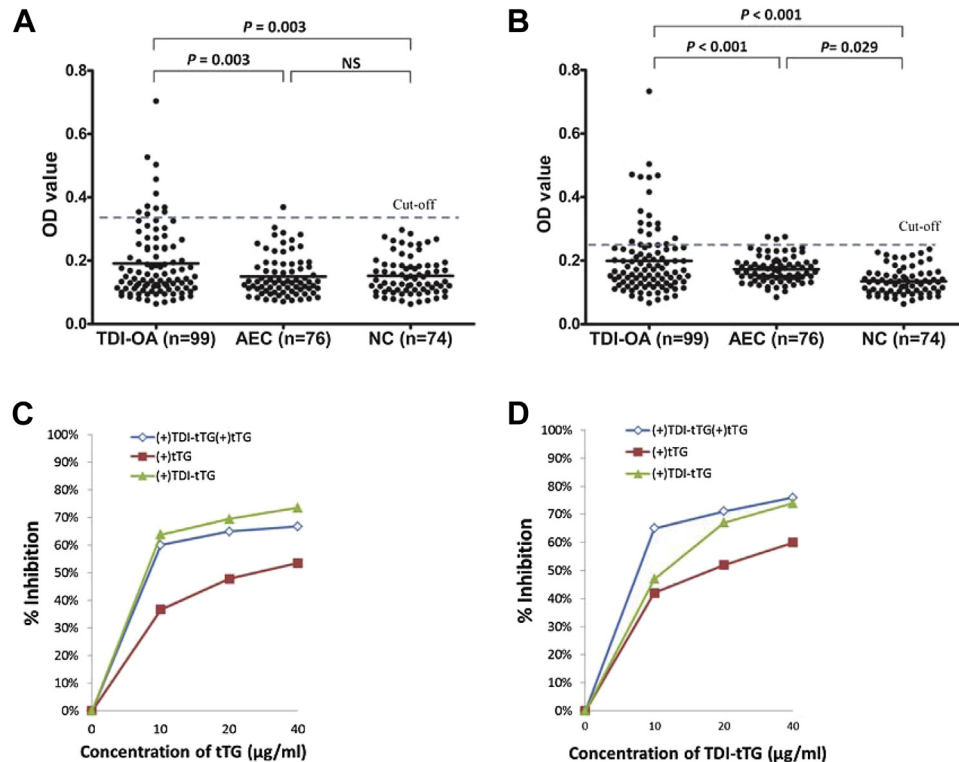


Figure 2. Detection of serum specific IgG antibody to (A) tTG and (B) TDI-tTG in 3 study groups. Horizontal dashed lines indicate the cutoff values (mean + 3-fold SD of NC group), and horizontal solid lines in the middle of the dot plots indicate the mean OD value of each group. The assays were performed in triplicate. Representative data of 3 experiments are shown in the graphs. *P* values were obtained by the Student *t* test to compare mean OD values among the 3 groups. Competitive enzyme-linked immunosorbent assay inhibition tests for specific IgG to (C) tTG and (D) TDI-tTG using 3 different serum samples: (+)TDI-tTG(+)tTG, serum sample with high level of specific IgG to TDI-tTG and tTG; (+)tTG, serum sample with a high level of specific IgG to tTG (but not to TDI-tTG); (+)TDI-tTG, serum sample with high level of specific IgG to TDI-tTG (but not to tTG). AEC, asymptomatic exposed control; NC, normal control; OA, occupational asthma; OD, optical density; TDI, toluene diisocyanate; tTG, tissue transglutaminase.

of the AEC group (11.65 ± 7.20 years). The AEC group had a significantly higher prevalence of smoking subjects (62.5%) than the TDI-OA (33.8%) and asthma (30.4%) groups. The prevalences of sIgG and sIgE to TDI-HSA in the TDI-OA group (25.3% and 23.9%, respectively) were significantly higher than those in the AEC (10.5% and 3.9%) and NC (0%, 0%) groups. The prevalence of sIgG to CK19 in the TDI-OA group (17.5%) was significantly higher than those in the 3 control groups ($P = .001$).

TDI-tTG Conjugate Production and Serum sIgG Antibody to tTG and TDI-tTG

The binding of TDI to tTG was identified by different migrations between tTG and TDI-tTG on a native gel (Fig 1). In a time-dependent manner, the migrations of TDI-tTG bands after 3, 5, 10,

20, and 30 minutes of incubation were not remarkably different. However, the density of TDI-tTG bands decreased after 10 minutes of incubation, which may be due to the aggregation of TDI-tTG conjugates. Thus, the conjugate produced with 3 minutes of incubation was selected for further tests.

Figure 2A and B show the levels of serum sIgG to tTG and TDI-tTG. The mean optical density values of sIgG to tTG and TDI-tTG in the TDI-OA group (0.191 ± 0.11 and 0.199 ± 0.12 , respectively) were significantly higher than those in the 2 control groups (0.15 ± 0.06 and 0.173 ± 0.04 in AEC group, 0.151 ± 0.06 and 0.135 ± 0.04 in NC group, $P < .05$ for all groups). In addition, a positive correlation in the sIgG level between tTG and TDI-tTG ($R^2 = 0.639$; $P < .001$) in TDI-exposed subjects was observed.

Table 2 presents the prevalence of serum sIgG to tTG and TDI-tTG in the 4 study groups that included 208 adult patients with asthma as disease controls. The prevalences of sIgG to tTG and TDI-tTG in the TDI-OA group (11.1% and 17.2%, respectively) were significantly higher than those in the AEC group (1.3% and 3.9%, $P < .05$) and patients with asthma (4.3% and 7.7%, $P < .05$). No subject in the NC group had sIgG to tTG and TDI-tTG.

Binding specificity was confirmed by ELISA inhibition tests applied to 3 serum samples (Fig 2C, D). Dose-dependent inhibitions greater than 50% were observed in all sera incubated with tTG or TDI-tTG. In addition, greater inhibitions were noted in sera with a high sIgG to TDI-tTG compared with those of sera with high sIgG to tTG.

Table 2

Prevalence of sIgG antibodies to tTG and TDI-tTG conjugate in TDI-OA, AEC, asthma, and NC groups

	TDI-OA (n = 99)	AEC (n = 76)	Asthma (n = 208)	NC (n = 74)	P value		
						TDI-OA vs AEC	TDI-OA vs asthma
sIgG to tTG ^a	11 (11.1) ^b	1 (1.3)	9 (4.3)	0 (0)	.013	.024	.223
sIgG to TDI-tTG ^a	17 (17.2) ^c	3 (3.9)	16 (7.7) ^a	0 (0)	.007	.012	.263

Abbreviations: AEC, asymptomatic exposed controls; NC, unexposed healthy controls; sIgG, serum specific IgG antibody; TDI-OA, patients with toluene diisocyanate-induced occupational asthma; TDI-tTG, conjugate of toluene diisocyanate and tissue transglutaminase; tTG, tissue transglutaminase.

^aData are presented as prevalence (percentage); *P* values were obtained by χ^2 test.

^b $P < .05$ vs NC group.

^c $P < .001$ vs NC group.

Serum sIgG to CK19 and Serum Levels of Cytokines according to sIgG Response to tTG and TDI-tTG

Table 3 presents a comparison of clinical and immunologic features and serum levels of various cytokines for TDI-exposed

Table 3

Comparison of clinical and immunologic features of 175 TDI-exposed subjects according to presence of sIgG to tTG and sIgG to TDI-tTG conjugate

	sIgG to tTG alone			sIgG to TDI-tTG conjugate		
	Positive (n = 12)	Negative (n = 163)	P value	Positive (n = 20)	Negative (n = 155)	P value
Age (y) ^a	40.0 ± 7.9	41.6 ± 9.4	NS	44.4 ± 7.7	41.1 ± 9.4	NS
Men ^b	9/12 (75)	118/162 (72.8)	NS	13/19 (68.4)	114/155 (73.5)	NS
Atopy ^{b,c}	3/9 (33.3)	33/71 (46.5)	NS	6/13 (46.2)	30/67 (44.8)	NS
FEV ₁ (%) ^{a,c}	81.58 ± 25.5	85.9 ± 21.9	NS	82.2 ± 25.8	85.9 ± 21.8	NS
Total IgE (kU/L) ^{a,d}	0.425 ± 0.813	0.304 ± 0.513	NS	0.362 ± 0.704	0.308 ± 0.522	NS
sIgG to CK19 ^{b,d}	2/11 (18.2)	16/162 (9.9)	NS	5/20 (25)	13/153 (8.5)	.039
TGF-β1 (ng/mL) ^{a,d}	36.9 ± 27.61	34.88 ± 37.97	NS	43.4 ± 18.18	33.94 ± 38.99	.032
TIMP-1 (ng/mL) ^{a,d}	71.61 ± 69.5	60.03 ± 54.25	NS	81.69 ± 51.3	58.06 ± 55.39	.003
MMP-9 (ng/mL) ^{a,d}	113.83 ± 107.83	192.72 ± 253.55	NS	206.15 ± 161.67	184.88 ± 256.19	NS
MPO (ng/mL) ^{a,d}	316.92 ± 650.84	124.9 ± 167.6	NS	240.51 ± 507.37	124.85 ± 171	NS
IL-8 (pg/mL) ^{a,d}	61.4 ± 105.19	57.94 ± 142.60	NS	49.06 ± 82.31	59.36 ± 146.04	NS
VEGF (pg/mL) ^{a,d}	403.58 ± 276.5	278.59 ± 222.98	NS	304.16 ± 246.82	284.17 ± 225.79	NS

Abbreviations: CK19, cytokeratin 19; FEV₁, forced expiratory volume in 1 second; IL-8, interleukin-8; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; NS, not significant; PC20, concentration of methacholine required to produce a 20% decrease in forced expiratory volume in 1 second; sIgE, serum specific IgE antibody; sIgG, serum specific IgG antibody; TDI-tTG, conjugate of toluene diisocyanate and tissue transglutaminase; TGF-β1, transforming growth factor-β1; TIMP-1, tissue inhibitor of metalloproteinase-1; tTG, tissue transglutaminase; VEGF, vascular endothelial growth factor.

^aData are presented as mean ± SD; P values were obtained by Mann-Whitney U test.

^bData are presented as prevalence (percentage); P values were obtained by Fisher exact test.

^cData from in vivo tests.

^dData from in vitro experiments.

subjects according to the responses of sIgG to tTG and TDI-tTG. Subjects with a positive response of sIgG to TDI-tTG had a significantly higher prevalence of serum sIgG to CK19 (25%) and higher serum levels of TGF-β1 (43.4 ± 18.18 pg/mL) and TIMP-1 (81.69 ± 51.3 ng/mL) compared with those without sIgG to TDI-tTG ($P < .05$ for all parameters).

Diagnostic Predictive Value of Serum sIgG to tTG and TDI-tTG Conjugate for Diagnosing TDI-OA

To distinguish TDI-OA from TDI exposure, the sensitivities of sIgG to tTG and TDI-tTG were found to be 11.1% and 17.2%, respectively; however, the sensitivity of sIgG and sIgE antibodies to TDI-HSA were 25.3% and 23.9%, respectively (Table 4). The combination of sIgG to TDI-tTG testing with sIgG to TDI-HSA increased the sensitivity (17.2% to 42.5%) and negative predictive value (47.1% to 56.5%) for diagnosing TDI-OA.

tTG and TDI-tTG Enhance IL-8 Production from HAECs

A549 cells were incubated with different concentrations of tTG, TDI-tTG, and TDI-HSA to evaluate the effects of tTG and TDI conjugates on IL-8 production from HAECs. The treatments did not affect cell viability, which was investigated by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assays

Table 4

Diagnostic predictive value of sIgG antibodies to tTG, TDI-tTG conjugate, and the combination of sIgG and sIgE antibodies to TDI-HSA conjugate confirmed by TDI bronchoprovocation test results in 175 TDI-exposed subjects

Serum specific antibody	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
sIgG to TDI-tTG and sIgG to TDI-HSA	42.5	85.5	77.1	56.5
sIgG to TDI-tTG and sIgE to TDI-HSA	38.6	92.1	85	56.5
sIgG to TDI-HSA	25.3	89.5	73.3	51.1
sIgE to TDI-HSA	23.9	96.1	87.5	52.1
sIgG to tTG and sIgG to TDI-tTG	19.2	94.7	82.6	47.4
sIgG to TDI-tTG	17.2	96.1	85	47.1
sIgG to tTG	11.1	98.7	91.7	46.0

Abbreviations: NPV, negative predictive value; PPV, positive predictive value; sIgE, serum specific IgE antibody; sIgG, serum specific IgG antibody; TDI-HSA, toluene diisocyanate conjugated to human serum albumin; TDI-tTG, conjugate of toluene diisocyanate and tissue transglutaminase; tTG, tissue transglutaminase.

(data not shown). The level of IL-8 measured from supernatants increased in a dose-dependent manner and the statistical significance was obtained with 200 μg/mL of TDI-HSA and 100 μg/mL of tTG and TDI-tTG conjugate (Fig 3). No significant differences were noted between the tTG-treated and TDI-tTG-treated groups.

Discussion

The authors previously reported a high prevalence of sIgG to tTG in sera of patients with TDI-OA using a commercial ELISA kit.¹⁰ This study replicated those findings to show a significantly higher prevalence of sIgG to tTG using human recombinant tTG and the authors' homemade ELISA system in the sera of patients with TDI-OA compared with the control groups. Based on these findings, the authors hypothesized that TDI could bind to tTG located in airway epithelial cells as a carrier protein to form a TDI-tTG conjugate, which would elicit immune responses to produce sIgG antibodies. The authors succeeded in producing the TDI-tTG conjugate at the first try, which was confirmed by a native gel, ELISA, and ELISA inhibition tests. Inhibitions greater than 50% in a dose-dependent manner observed in ELISA inhibition tests (Fig 2C, D), as reported elsewhere,¹⁷ could confirm the specificities of the ELISA assays performed in the present study. Higher levels and the prevalence of sIgG to TDI-tTG were found in the TDI-OA group compared with the 3 control groups when compared among the 4 study groups. A higher prevalence of sIgG to TDI-tTG was noted when compared between the tTG and TDI-tTG conjugate; however, it did not reach statistical significance. The findings suggested 2 potential mechanisms for TDI-OA pathogenesis. First, TDI binds to tTG to form a TDI-tTG conjugate that can generate new epitopes presented to the immune system and induce sIgG production, as described by Kim et al¹⁸ who reported that the haptenation of host proteins with isocyanates could lead to the recognition of self-proteins by the immune system. In addition, tTG can generate new epitopes by crosslinking.¹⁰ Second, new epitopes on tTG could be produced by post-translational modifications of the protein by some factors such as ROS without haptenation. ROS exists in the environment and is produced by TDI in exposed airway epithelial cells; in addition, it acts as an oxidative post-translational modifying factor to convert host proteins into autoantigens.^{10,19} Therefore, TDI exposure may modify tTG located in HAECs that can elicit sIgG responses to tTG and TDI-tTG and induce airway inflammation.

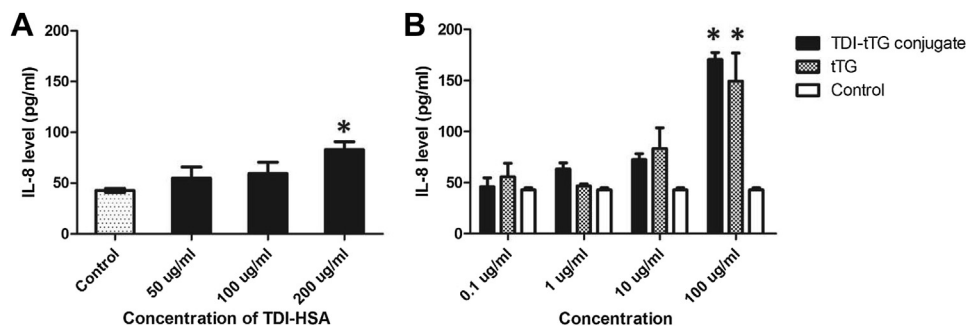


Figure 3. Effects of TDI conjugates and tTG on IL-8 production from airway epithelial cells. A549 cells were treated with different concentrations of (A) TDI-HSA conjugate or (B) tTG and TDI-tTG conjugate. Controls were conducted by treating cells with serum-free medium. Supernatants were collected and IL-8 levels were measured by enzyme-linked immunosorbent assay. Data are presented as mean $\pm$ SEM of 3 independent experiments. *P* values were obtained by 1-way analysis of variance. **P* < .05 vs control. IL-8, interleukin-8; TDI, toluene diisocyanate; tTG, tissue transglutaminase.

The higher prevalence of sIgG to CK19 in patients with TDI-OA and a potential crosslink between CK19 and tTG have been noted.^{10,16} CK in HAECS could bind to hexamethylene diisocyanate and produce the conjugate²; hence, CK19 may interact with TDI or tTG. In the present study, a significant association was observed between sIgG to CK19 and sIgG to TDI-tTG. It has been reported that the post-translational enzymatic activity of tTG could modify the structure of various proteins.^{5,6,20} Therefore, the authors speculate that the sIgG response to CK19 may be derived from the haptenation of CK19 with TDI and/or from the modification of CK19 biochemical characteristics by TDI-activated tTG. The authors also detected sIgG to tTG and TDI-tTG in a few TDI-unexposed patients with asthma. The findings can be explained by ROS-induced activation and modification of tTG in HAECS because it has been widely accepted that more ROS is produced endogenously in inflammatory cells from patients with asthma than from healthy controls; in addition, patients with asthma are exposed to exogenous ROS from the environment.^{21,22}

Previous studies have indicated that overexpressed tTG in ovalbumin-sensitized mice could enhance the production of immunoglobulins by B lymphocytes; in addition, tTG could involve the recruitment of eosinophils, IL-33 expression, and T-helper type 2 and 17 differentiation in asthma pathogenesis.^{9,23} Therefore, TDI-induced ROS-activated tTG may play a pivotal role in immune responses that include the production of autoantibodies to various host proteins, immune cell activations, and differentiations.

Previous studies have shown that TDI exposure can upregulate TGF- β 1 expression.^{24,25} In the present study, higher serum levels of TGF- β 1 and TIMP-1 were observed in the sera of TDI-exposed subjects who had a high serum level of sIgG to TDI-tTG conjugate. Tissue TG and TGF- β 1 have a mutual interaction in which the increased expression of tTG can upregulate the expression of TGF- β 1²⁶; in addition, activated TGF- β 1 enhances the synthesis of tTG as a feedback loop.^{27,28} TGF- β 1 also upregulates the expression of TIMP-1, a cytokine involved in tissue remodeling.^{29–31} The findings suggest that TDI-activated tTG may be associated with the production of inflammatory and remodeling cytokines.

Interleukin-8 secreted from airway epithelial cells plays an important role in TDI-induced airway inflammation by recruiting neutrophils to the airways.³² Numerous studies have indicated that the TDI-HSA conjugate can induce IL-8 production from HAECS.^{33,34} Therefore, levels of IL-8 secreted from tTG- and TDI-tTG-treated HAECS were evaluated and tTG and TDI-tTG were found to increase IL-8 production. Similar to the TDI-HSA conjugate, the increased production of IL-8 by TDI-tTG could be explained by TDI-induced ROS.^{10,22,35} In addition, various in vitro studies have observed that tTG increases the activity of secretory phospholipase A2,^{36–38} and that several isoforms of secretory phospholipase A2 induce the

production of IL-8 from airway epithelial cells and lung macrophages.^{39,40} Tissue TG and TDI-tTG conjugate might affect the production of IL-8 from HAECS (by which tTG contributes to the airway inflammation of TDI-OA); however, this mechanism needs to be investigated further.

The authors also evaluated the diagnostic values of serum sIgG to tTG and TDI-tTG for the diagnosis of TDI-OA. The sensitivities of sIgG to tTG and TDI-tTG detection were inadequate; however, these tests had high specificities to distinguish patients with TDI-OA among those with TDI exposure. The combination of sIgG to the TDI-tTG assay with sIgE or sIgG to TDI-HSA increased the sensitivities (38.6% with 85% positive predictive value, 42.8% with 72.1% positive predictive value, respectively) for diagnostic tests. The authors could not identify any predisposing factor for a high sIgG response to TDI-tTG (Table 3); therefore, these serologic diagnostic methods need to be modified, improved, and standardized.

In TDI-exposed subjects, the prevalence of smokers was significantly higher in the AEC group than in the TDI-OA group (Table 1). This finding was consistent with the previous study reporting that smoking workers (particularly currently smoking subjects) were more prevalent among asymptomatic TDI-exposed workers than among those with TDI-induced asthma.⁴¹ Furthermore, other groups have observed a difference in response to the TDI bronchial challenge test between smokers and nonsmokers⁴² and that smoking attenuated increased eosinophilia in asthma.⁴³ These findings may suggest a protective effect of smoking against TDI-induced asthma; however, further studies are needed to investigate the role of smoking in patients with TDI-OA.

There were limitations in analyzing the biochemical characteristics of TDI-tTG, such as the TDI binding sites and the binding ratio of TDI to tTG. In addition, the TDI-tTG conjugate was produced in vitro and has not been identified in human tissue. Further studies are required to investigate the cellular roles of TDI-tTG in the airway inflammatory mechanism of TDI-OA.

In conclusion, the authors observed a novel mechanism for TDI-OA pathogenesis in which TDI binds to tTG to form a conjugate that can induce serum sIgG antibody production and increase airway inflammation and airway remodeling in patients with TDI-OA.

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