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Cytokine Secretion Patterns of T Cells Responding to Haptenized-human Serum Albumin in Toluene Diisocyanate (TDI)-induced Asthma Patients

Our understanding immune response mechanisms to chemical allergens has been limited. It was partly due to the nature of antigens, recognized by T cells, not being well characterized. In the present study, we examined a hypothesis that a reactive chemical allergen, toluene diisocyanate (TDI), react with autologous proteins, thereby inducing T cell responses to the modified self protein in vivo. TDI-human serum albumin (HSA) conjugates were prepared and the presence of antigenic epitopes on the TDI-HSA conjugate was confirmed by IgE ELISA. We examined proliferative and cytokine production responses in TDI-induced asthma patients using the TDI-HSA conjugate as an antigen. Although proliferative responses of peripheral blood mononuclear cells (PBMCs) were not detected, production of IFN-γ was observed in both PBMC and T cell lines obtained from some newly-diagnosed patients by ELISA. Mitogen-inducible IL-4 production was also detected in some T cell lines. Results of this study may have two implications. One is that presentation of haptenized-self protein to the immune system may induce activation of T cells. The other is that T cells responding to this modified self protein may play a role in the pathogenesis of the chemical allergen-induced asthma by producing cytokines such as IFN-γ and possibly IL-4.

Key Words : *Haptens; Toluene 2,4-diisocyanate (TDI); Allergen, chemical; T-lymphocytes; Cytokines*

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INTRODUCTION

A group of low-molecular weight chemical compounds containing -N=C=O groups has been shown to cause allergic sensitization of the respiratory tract (1). Among these chemicals, toluene diisocyanate (TDI), the most common cause of occupational asthma, has been reported to induce specific humoral immunity as a hapten. Whereas the presence of specific IgE and IgG in the sera has been shown by a number of investigators (2-7), specific T cell responses for this chemical allergen has not been sufficiently studied. Considering that T cells play a critical role in B cell activation and isotype switching, it is likely that exposure to a chemical allergen may induce activation of T cells. In addition to the role as a helper T cell, activated T cells may participate directly in the initiation and propagation of allergic inflammation through secretion of various cytokines. This possibility can be supported by a finding that T cells producing a variety of cytokines were recruited in the airway mucosa following a challenge with TDI (8).

Cytokine profiles during the immune response against an antigen are thought to be determined by multiple factors including the nature of the antigen, types of antigen presenting cells and genetic factors (9). Upon antigenic stimulation CD4+ T cells have been shown to differentiate into distinct subsets characterized by distinct cytokine secretion patterns. Recent data have demonstrated that not only CD4+ $\alpha\beta$ TCR T cells, but also CD8+ $\alpha\beta$ TCR T cells and $\gamma\delta$ TCR T cells can demonstrate the heterogeneity of cytokine secretion patterns with respect to IL-4 and IFN-γ (10). Based on the dichotomy of cytokine production by T cell clones, cytokines such as IFN-γ and IL-2 are called Th1 type cytokines, while cytokines such as IL-4, IL-5 and IL-10 are called Th2 type cytokines (9). While Th2 type cytokines have been suggested to play an important role in atopic asthma (11-13), little is known about the roles of cytokines secreted

by T cells in the chemical allergen-induced airway hypersensitivity.

In order to act as an antigen recognized by T cells, low molecular weight haptens must first react with autologous or heterologous proteins. Actually, a recent report showed presence of albumin adducts in plasma from workers exposed to TDI (14). Therefore, it can be hypothesized that before presented to the immune system, inhaled TDI would interact with autologous self proteins in the body through covalent bonding with its highly reactive isocyanate groups. To assess responses of T cells to the haptenized self protein, we prepared TDI-human serum albumin (HSA) conjugates and examined proliferative responses of T cells and cytokine secretion profiles in the TDI-induced asthma patients.

MATERIALS AND METHODS

Subjects and reagents

Subjects studied were 4 newly-diagnosed TDI-induced OA patients (T27-T30), 14 follow-up patients who had avoided exposure to TDI after the initial diagnosis (1 month to 7 years) and 8 control healthy individuals who had no history of exposure to TDI. All patients were diagnosed as having TDI-induced occupational asthma proven by TDI-bronchial challenge test (6). All reagents for preparation of TDI-HSA conjugates were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of TDI-HSA conjugates

TDI-HSA conjugates were prepared by modifying the Tse and Pesce's method (15) as described in a previous report (16). Briefly, TDI (toluene-2,4-diisocyanate) was added to 1% human serum albumin in phosphate buffered saline with constant stirring. Aliquots were taken after 5, 10, 20, 30, and 40 min and 2 M ammonium carbonate was added to each aliquot to terminate reactions. All samples were centrifuged at 3,000 *g* for 20 min at room temperature, dialyzed in 0.1 M ammonium carbonate for 3 days, precipitated with trichloroacetic acid, redissolved in 1 M sodium hydroxide, and then extensively dialyzed in distilled water for 3 days.

Determination of amount of TDI bound to HSA

Degrees of substitution were determined by a modified Gutmann assay (17). Briefly, the assay was performed on 50 μl of aliquot samples to which 50 μl of concentrated HCl, 50 μl of distilled water and 0.2 ml of 0.029 M sodium nitrate were added. 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 5min, 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. Protein content of a conjugated sample was determined by Lowry method (18) using bovine serum albumin as a standard.

IgE ELISA

Specific binding of a patient's serum IgE to prepared TDI-HSA conjugates was examined by IgE ELISA as described in a previous report (16). Briefly, serum was added to each well of ELISA plates (Costar, Cambridge, MA) coated with 10 μg of a TDI-HSA conjugate. After overnight incubation at 37℃, plates were washed with PBST (PBS containing 0.1% Tween 20 and 0.2% sodium azide). After biotinylated anti-human IgE (Sigma Chemical Co., BA-3040) diluted to 1:1000 with 5% BSA-PBST was added, plates were incubated for 3hr 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 1 hr at 37℃, plates were washed and substrate solution (p-nitrophenyl phosphate) was added. Optical density at 405 nm was determined using an ELISA reader (Molecular Devices CO., USA).

Culture media

The culture medium used was RPMI1640 supplemented with 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin (all from GIBCOBRL Lab., Grand Island, NY) and 10% human AB serum (Sigma Chemical Co., St. Louis, MO). Recombinant human IL-2 (rhIL-2) was purchased from Sigma Chemical Co. Phytohemagglutinin (PHA; Sigma Chemical Co.) was used in a concentration of 1 μg/ml.

Proliferative assay

To measure proliferative responses of PBMC to stimulation with a TDI-HSA conjugate, cells (2×10^5/well) suspended in the complete medium were cultured for 6 days in the presence of TDI-HSA (10 μg/ml) in 96-well U bottom tissue culture plates (Costar, Cambridge, MA). The cultures were pulsed during the last 18 hrs with 1 μCi/well of ^{3}H-thymidine (New England Nuclear, Boston, MA). A positive PHA control in triplicate was run parallel and all assays were carried out in triplicate or

six replicates. After harvesting in a cell harvester (CHI, Insel Co. Ltd., England), the amount of cell-incorporated radioactivity was measured in a liquid scintillation counter (Pharmacia LKB, Uppsala, Sweden). Results were expressed as stimulation index (SI); SI is the ratio between the mean cpm in the presence of antigen and mean cpm in the absence of antigen.

Preparation of TDI-HSA-reactive T cell lines

Peripheral blood mononuclear cells (PBMC) were separated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation. Using 24 well culture plates (Costar, Cambridge, MA), cells (2×10^6/well) in the complete medium were cultured in the presence of TDI-HSA (10 μg/ml) for 7 days at 37℃ in a humidified 5% CO_2 incubator. Then TDI-HSA-reactive T cells were preferentially expanded for 7 days by adding rhIL-2 (10 U/ml). For cytokine assays, these T cell lines (1×10^5/well) were stimulated with TDI-HSA (10 μg/ml) in the presence of irradiated (3,000 Rad; gamma cell 3000 Elan; Nordion, Kanata, Ontario) autologous PBMC (2×10^5/well) as feeder/antigen presenting cells.

Cytokine assay

Supernatants of the antigen-stimulated PBMC or T cell line cultures were collected after 4 days of incubation, and assays for IFN-γ and IL-4 were performed using human cytokine ELISA kits (Endogen, Woburn, MA). The assay sensitivities of IFN-γ and IL-4 were 10.2 and 2.0 pg/ml, respectively.

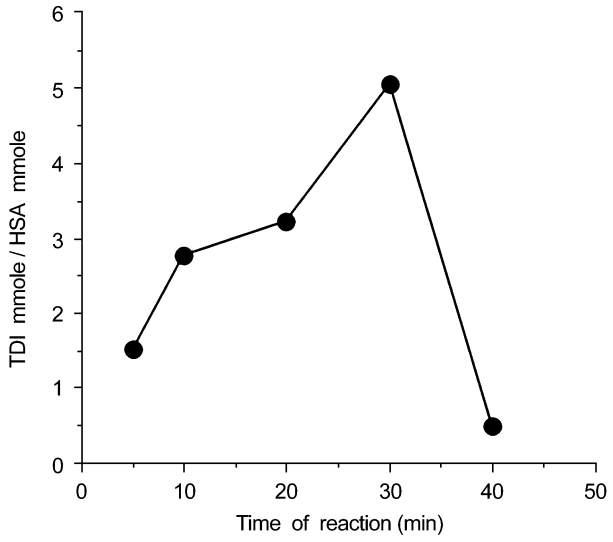

Fig. 1. Amount of TDI bound to HSA. Degrees of substitution for different TDI-HSA conjugates, prepared at different reaction times, were determined by modified Gutmann assay.

RESULTS

TDI-HSA conjugates

To stimulate TDI-HSA-reactive T cells, TDI-HSA conjugates were prepared. As Fig. 1 shows the amount of TDI bound to HSA increased as reaction time was increased up to 30 min. When reaction time was longer than 30 min, only a minimal amount of protein was recovered from samples. To ensure that our TDI-HSA conjugates have immunogenic epitopes, binding of specific IgE to the various TDI-HSA conjugates was examined by IgE ELISA. Using a known positive serum obtained from a TDI-induced asthma patient, binding of specific IgE to the TDI-conjugates was detected (Fig. 2). Since TDI-HSA conjugate reacted for 30 min was found to contain the highest number of TDI molecule per HSA molecule (Fig. 1), we decided to use this TDI-HSA conjugate as an antigen for further studies.

Proliferative responses and cytokine production of PBMC

To investigate whether there is any responses of T cells to the TDI-HSA, PBMC proliferation and production of IFN-γ and IL-4 in the presence of the TDI-HSA were

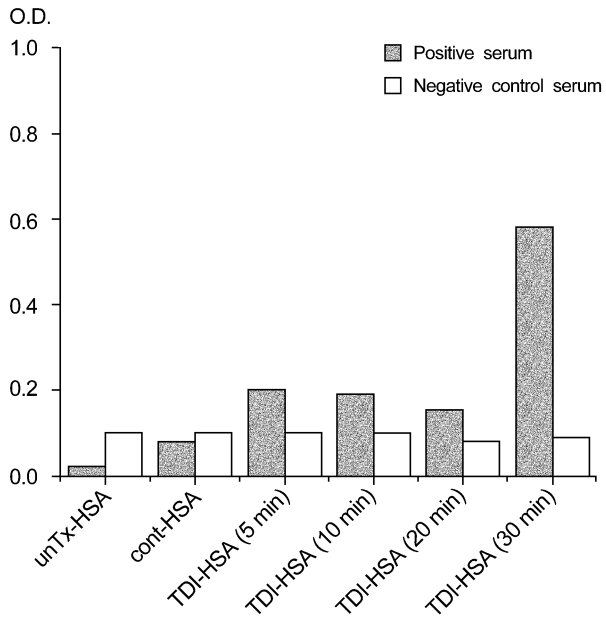

Fig. 2. Interactions of TDI-HSA conjugates with a known positive serum from a TDI-induced OA patient measured by IgE ELISA. TDI-HSA (5 min), TDI-HSA (10 min), TDI-HSA (20 min), TDI-HSA (30 min) represent TDI-HSA conjugates which reacted for 5, 10, 20 and 30 min, respectively. cont-HSA, HSA treated following exactly the same procedure except adding TDI; unTx-HSA, untreated HSA.

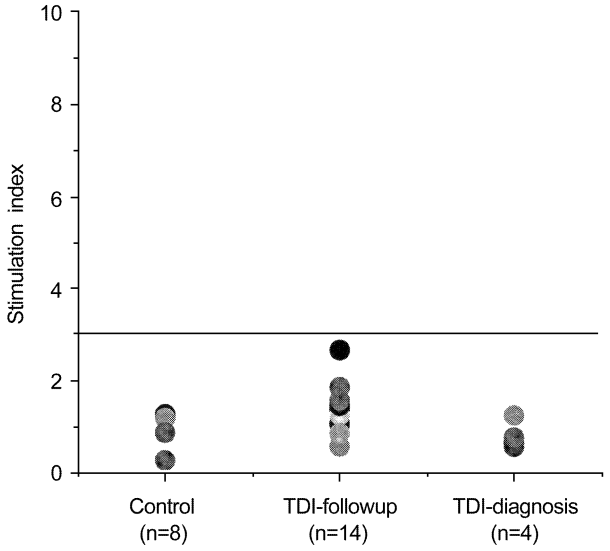

Fig. 3. Proliferative responses of PBMCs to TDI-HSA. PBMCs (2×10^5/well) were cultured for 6 days in the presence and absence of TDI-HSA (10 μg/ml). Cell proliferation was determined by ^{3}H-thymidine uptake over the final 18 hr of the culture.

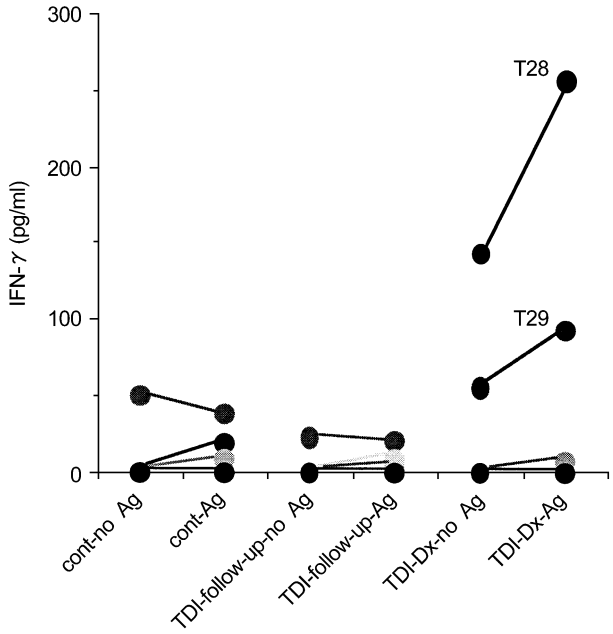

Fig. 4. Production of IFN-γ by PBMC stimulated with TDI-HSA. PBMCs (2×10^5/well), obtained from TDI-induced asthma patients (follow-up, n=14; newly diagnosed patients, n=4) and control individuals (n=8), were cultured for 4 days in the presence and absence of TDI-HSA (10 μg/mL). Culture supernatants were collected and assayed for IFN-γ using ELISA kits. It was noted that PBMCs from two newly-diagnosed patients (T28 & T29) showed increased levels of IFN-γ production in the presence of TDI-HSA compared with those in the absence of TDI-HSA. Cases showing same levels of IFN-γ production were overlapped and shown as a single circle.

examined. When values of stimulation index higher than three were considered significant, neither follow-up patients nor newly dignosed patients showed any significant level of proliferative response (Fig. 3). Although no proliferative response of PBMC was observed, production of IFN-γ by PBMC in the presence of the TDI-HSA was detected in some cases of newly diagnosed patients (Fig. 4). Meanwhile, production of IL-4 by PBMC was not detected in any cases (data not shown).

Cytokine production by T cell lines

To obtain a larger number of TDI-HSA-reactive T cells, primary T cell lines were generated by stimulation with the TDI-HSA and subsequent expansion of cells by rhIL-2. Although none of the T cell lines generated from PBMC of newly diagnosed patients proliferated in the presence of the TDI-HSA (data not shown), some T cell lines, especially T28 line from patient LB, produced IFN-γ in the presence of the TDI-HSA (Table 1). Meanwhile, it was noted that three out of four T cell lines produced IL-4 when stimulated with PHA (Table 1).

Table 1. Cytokine production by T cell lines in response to TDI-HSA

T cell lines*	Antigen	IFN-γ (pg/ml)	IL-4 (pg/ml)
T27	TDI-HSA	0	0
	PHA	1,281	2.5
T28	TDI-HSA	59	0
	PHA	3,152	0
T29	TDI-HSA	49	0
	PHA	2,900	2.8
T30	TDI-HSA	2	0
	PHA	3,310	7.8

* Each T cell line was prepared from PBMC of each individual of newly-diagnosed patients (T27-30) as described in Materials and Methods.

DISCUSSION

TDI, a known cause of respiratory hypersensitivity, has been suggested to be able to provoke immune responses. TDI-HSA conjugates have been used as antigens to detect TDI-specific IgE in the patients' sera and a number of reports have shown production of specific IgE in 10-40% of TDI-induced asthma patients (2-7). As far as we know, specific T cell responses to TDI-HSA conjugates have not been reported. In the present study, various TDI-HSA conjugates were prepared in our laboratory and the TDI-HSA conjugate containing the highest

number of TDI molecules was chosen to stimulate T cells. Although proliferative responses were not detected, production of cytokines by PBMC or T cell lines were observed. Results of the present study may have two implications. First, self proteins modified by reactive chemical allergens may be presented to the immune system to induce activation and differentiation of T cells. Thus, activation of T cells specific for TDI-HSA may be induced by chronic exposure to TDI. Second, cytokine profiles responding to TDI-HSA seemed to include IFN-γ and possibly IL-4.

T cells may play an important role in the chemical allergen-induced inflammation of the airway. After challenging with TDI, an increase in CD8+ cells and eosinophils was observed in the peripheral blood of subjects who showed asthmatic reactions (19) and a significant increase in the number of CD25+ cells and eosinophils was observed in the bronchial mucosa (20). Further investigations on phenotypes of T cells infiltrating into TDI-challenged bronchial mucosa revealed presence of both CD4+ and CD8+ T cells and that majority of T cells were CD8+ (8). Pathogenic roles of lymphoid cells were suggested by a report showing adoptive transfer of airway hypersensitivity by lymphoid cells from TDI-sensitized mice in the absence of TDI-specific IgE (21). Meanwhile, adoptive transfer of hapten-induced inflammation by T cells has been shown in an animal model of inflammatory bowel disease induced by rectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS), in which haptenized colonic proteins seemed to be targets of T cell responses (22).

What TDI antigen looks like is unknown. Since TDI is a low-molecular weight chemical (MW=174.15), it is unlikely that TDI itself is the target antigen of T cells. Following inhalation exposure to ^{14}C-labeled TDI, rapid accumulation of inhaled TDI in the lung was shown in animal models (23). Considering TDI contains highly reactive isocyanates, it can be expected that TDI would combine covalently with body protein to form TDI-protein conjugates. These modified self proteins may become potentially accessible to antigen presenting cells (APCs) in the lung. If lung APCs took up the TDI-protein conjugates, they would process and present chemically modified-peptides to T cells in association with MHC molecules. Since TDI could alter the structure of peptide epitopes, it can be speculated that processed TDI-protein conjugates may be recognized by T cells as a foreign antigen and specific immune responses to the altered proteins may develop. It is also possible that TDI could directly modify the structures of MHC molecules on the surface of APCs. Recently, an association between TDI-induced asthma and amino acid residue 57 of HLA-DQ B1 was reported (24). How this particular MHC allele involved in the pathogenesis of TDI-induced asthma is unknown.

Cytokine secretion patterns provoked by chemical allergens have not been extensively studied. Cytokine profiles of T cells responding to PHA stimulation were examined among T cell clones derived from bronchial biopsies of TDI-induced asthma patients (8). Of CD8+ T cell clones, all clones produced IFN-γ and 44% of them produced IL-5. Of CD4+ clones, 35% were Th1, 48% were Th0 and 17% were Th2 (8). Thus, it is likely that heterogenous T cells capable of secreting various kinds of cytokines infiltrated the bronchial mucosa following exposure to TDI. Dearman and his colleagues reported differences in cytokine production patterns between respiratory (trimellitic anhydride; TMA) and contact chemical allergens (oxazolone) in mice chronically exposed to these chemical allergens (25, 26). In these studies, ConA-stimulated regional lymph node cells (LNCs) exposed to TMA produced IL-4, whereas those LNCs exposed to oxazolone did not. In contrast, a significantly larger amount of IFN-γ was produced by oxazolone-activated LNCs compared with TMA-activated LNCs. Further studies revealed that high levels of mitogen-inducible IL-4 and spontaneous IL-10 secretion by LNCs from mice sensitized to respiratory chemical allergens such as TMA or diphenylmethane diisocyanate were dependent upon the presence of CD4+ T cells (27). Production of the Th2 type cytokines such as IL-4 and IL-10 by TDI-sensitized lymph node cells was also reported (28). The data from several reports by Dearman et al. suggest that exposure to chemical allergens such as TDI can result in effective sensitization of the immune system. Target antigens recognized by T cells, however, have not been elucidated.

Mitogen-inducible IL-4 secretion by the T cell lines may suggest that chronic exposure to TDI could induce productions of Th2 type cytokines in vivo. A recent report showed expression of IL-4 and IL-5 in the cells of bronchial biopsies from TDI-induced asthma patients using immunohistochemistry (29). Activated T cells specific for modified self proteins may be partly responsible for secretion of these cytokines. Cloning of TDI-HSA-reactive T cells will reveal the phenotypes and functional characteristics of these T cells.

Pathogenic roles of TDI-HSA-reactive T cells are totally unknown. IL-5 produced by these T cells may play a role in the recruitment and activation of eosinophils in the airway (30). IL-4 may induce B cell isotype switching to IgE antibody production (31). We could also observe IFN-γ production by T cells from some newly-diagnosed patients. Although IFN-γ has generally been regarded as an anti-allergic cytokine, IFN-γ has a variety of pro-inflammatory activities which could contribute to the

airway hypersensitivity. For example, IFN-γ increases expression of ICAM-1 (32) and an inducible form of nitric oxide synthase (33).

In the present study, we could observe production of IFN-γ by T cells responding to a self protein modified by a chemical allergen. Further studies will reveal whether or how T cells specific for the modified self proteins play a role in the pathogenesis of the chemical allergen-induced asthma.

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