Ovalbumin Fused with Diphtheria Toxin Protects Mice from Ovalbumin Induced Anaphylactic Shock

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For those with allergy, vaccination with a specific allergen has often been used as a major therapeutic measure. However, the universal application of this technique in clinics has been restricted due to its low success rates and the risk of active systemic anaphylactic shock (ASAS). In this regard, we constructed a fusion protein (OVA-DT), ovalbumin (OVA) fused with diphtheria toxin protein (DT), which may exert a specific cytotoxicity to cells bearing OVA-specific IgE. Its therapeutic effect was evaluated in mice (BALB/c) sensitized with OVA (Ox-mice). OVA challenges to the OVA-sensitized mice (Ox-mice) caused ASAS to death within 30 min, but OVA-DT treatment afforded mice complete protection. When OVA-DT was treated to the Ox-mice, none showed the signs of ASAS when re-challenged 48h after the treatment. OVA-DT itself was not found to be toxic or allergenic in normal mice. The effect of OVA-DT on the biological functions of mast cells was also studied. Binding of OVA-DT to OVA-specific IgE bearing mast cells and the inhibition of histamine release from these cells were observed. In addition, OVA-DT treatment inhibited the proliferation of OVA-specific B cells in mice. In Ox-mice treated with OVA-DT, levels of anti-OVA IgG2a in serum and the production of IFN-γ by splenic lymphocytes were found to increase, but the production of IL-4 by these cells decreased. Re-direction of cytokine profiles from OVA-specific Th2 to OVA-specific Th1 is suggested.

These results indicate that OVA-DT can protect Ox-mice from ASAS due to OVA challenge, because it inactivates OVA-specific IgE-expressing cells, including mast cells and B cells.

Key words: Immunotherapy, fusion protein, spleen index, IL-4, IFN-γ, histamine, IgE

INTRODUCTION

Allergies are immunological disorders that are accompanied by various clinical symptoms, such as allergic asthma, rhinitis, conjunctivitis, and dermatitis. The therapeutic approach for allergy is mainly dependant on chemotherapy, which generally provides temporary relief and rarely, complete remission. Curtis et al (1900)¹ and Noon et al (1911)² developed a therapeutic vaccination with specific allergen (immunotherapy) for the permanent and complete treatment of allergy. Since then this method has been used as the major therapeutic measure. However, allergy therapy by vaccination requires long-term therapy (one to three years) since a specific antigen must be identified prior to the therapy and the patient must receive a specific allergen in increasing doses periodically. Furthermore, its low success rates and the risk of ASAS limits the universal application of this method in clinics.

Various attempts and alternative approaches had been made to develop safe complete therapies, but without significant results. These have included modifying allergens (allergoids),³⁴ and the use of immunologic adjuvants.⁵⁶ Recently, cytokines have been reported to be important factors in the regulation of immune responses, especially in allergic response,⁷ in which the major responding cells are known to be type 2 helper T lymphocytes, (Th2). Activation of Th2 cells triggers numerous events, including the induction of IgE switching factors, such as IL-4 and IL-13,

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which induce B cells to produce IgE. Activated Th2 also produces IL-5, which induces the proliferation of eosinophils and recruit them into the site of inflammation resulting in an allergic reaction. In this respect, for the purpose of allergy therapy, efforts are mainly focused on the induction of non-specific IFN-γ production in vitro, on the basis that it might enhance Th1 mediated cytokine production, which in turn might repress the function of Th2 mediated cytokines and IgE. In this regard, various means of enhancing Th1 induced IFN-γ and/or macrophage induced IL-12 have been examined, such as, the application of fusion proteins of IL-12 and allergen, produced by cDNA technology or by vaccination with plasmid containing allergen cDNA to induce IFN-γ production. These efforts have resulted in a partial reduction in the levels of allergen specific IgE produced by B cells. However, the possibility of ASAS remains since the complete elimination of cells involved in allergen specific IgE production cannot be expected using these methods.

An experimental therapy for allergy has been designed that involves eliminating the allergen specific IgE-expressing cells involved. This therapy is based on specifically killing the allergen specific IgE-expressing cells by using a cytotoxic protein fused with the allergen. The allergen-toxin fusion protein was prepared using OVA, which has been proven to be a fatal allergen, and Corynebacterium diphtheriae toxin (DT) as the cytotoxic protein. It has been well established that diphtheria toxin is a potent cell-killing agent and has been used commonly in preparations of immunotoxins for cancer therapy. The novel fusion protein, OVA-DT was expressed using recombinant genes transformed and produced in E. coli. As a result of this work, we are able to demonstrate that OVA-DT protects OVA sensitized mice from death due to anaphylactic shock caused by OVA challenge.

MATERIALS AND METHODS

Mice

Female BALB/c mice (6 and 7 wks. of age, 17-18 g/mouse) bred in specific pathogen free condition were obtained from the Korean Institute of Science and Technology (Denjeon, Korea) and housed in a pathogen-free animal facility at Yonsei University College of Medicine, Seoul, Korea.

Reagents

OVA, concanavalin A (ConA, Type IV) and lipopolysaccharide (LPS, L 4005) were purchased from the Sigma Chemical Co. (ST. Louis, MO, USA), and formalin-inactivated Bordetella pertussis was obtained from the Green-Cross Co. (Seoul, Korea).

Antibodies

Purified mouse IgE, IgM and IgG subclasses (IgG1, IgG2a and IgG3), purified monoclonal antibodies (mAbs) of rat anti-mouse IgE, IgM and IgG subclasses, and biotin-conjugated mAbs of rat anti-mouse-IgM and IgG subclasses were purchased from PharMingen (San Diego, CA, USA). Peroxidase-conjugated goat anti-biotin Ab and peroxidase substrate (o-phenylenediamine, OPD) were purchased from Sigma. Anti-diphtheria toxin Ab was prepared from rabbit immunized with diphtheria toxoid once a week for 4 weeks. FITC-conjugated goat anti-rabbit immunoglobulins (IgM and IgG) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ab were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). Anti-OVA Ab was prepared from rabbit immunized with mixture of OVA and complete Freund's adjuvant once a week for 4 weeks.

Plasmid constructions

The chicken OVA cDNA (pOv) and Corynebacterium diphtheriae toxin cDNA (pDT) were kindly provided by Michel M. Sanders (Dept. of Biochemistry, Minnesota University) and Jae-Ku Park (Dept. of Microbiology, National Institute of Health, Korea), respectively. The truncated form of DT containing the ADP-ribosylation catalytic domain and the transmembrane domain (117bp) was obtained by PCR with pDT as the template.
and the oligonucleotide primers: 5'-GGATCCGG CGCTGTATGGTGTGTGA-3' and 5'-CTGACG CGCCTGACCAGATTTCCGTC-3'. The PCR product was then subcloned into a pRSETA vector containing a T7 promoter (Invitrogen, Carlsbad, CA), which was named pRSETA/DT. The OVA insert fragment, which removed signal peptide (+484+1222, 738bp), was prepared by PCR with pOV1 as a template, and by using the following primers: 5'-GCTGCAAGATCCAGCGAGCAGCT-3' and 5'-GAATCCAGGGGAAACACACACTCTGC C-3'. The PCR product was then subcloned into a pRSETA vector, which was named pRSETA/OVA.

After an initial denaturation step of 7 min at 94°C, the DNA was amplified using 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, and re-extended at 72°C for an additional 10 min. Finally, the amplified OVA insert fragments were digested with PstI and EcoRI, and ligated into pRSETA/DT, which was named pRSETA/OVA- DT. The plasmids were transformed into E. coli BL21, and clones containing the correct insert were identified by agarose gel electrophoresis (Fig. 1).

Fig. 1. Plasmid construction of OVA-DT. The truncated form of DT was obtained by PCR with the oligonucleotide primers 5'-GGATCCGGCGTGTATGGTGTTGTA-3' and 5'-CTGACGCGCCCGCGTGTGTGTCGTC-3'. The PCR product was then subcloned into a pRSETA, which was named pRSETA/DT. The OVA insert fragment was prepared by PCR with the following primers: 5'-GCTGCAAGATCCAGCGAGCAGCT-3' and 5'-GAATCCAGGGGAAACACACACTCTGC C-3'. Finally, the amplified OVA insert fragment was digested and ligated into pRSETA/DT, which was named pRSETA/ OVA-DT.
Expression of recombinant proteins

BL21 transformed with pRSETA/OVA-DT fusion plasmids were grown until OD600 is 0.6. IPTG (isopropyl-β-D-thiogalactoside) was added to a final concentration of 1mM, and this was followed by additional growth for 2h. Purification of OVA-DT was performed using a Ni-NTA purification system (QIAGEN, Hilden, Germany). Briefly, the bacterial cells were harvested, and lysed in sonication buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and then centrifuged at 15,000 rpm for 30 min at 4°C. The pellet was suspended in solubilization buffer (8M urea, 0.1M NaH2PO4, and 0.01M Tris-Cl, pH 8.0) and incubated for 1 h with shaking at room temperature (RT). The resuspended pellet was then centrifuged at 15,000 rpm for 30 min at 4°C, and the supernatant mixed with Ni-NTA resin at 4°C for 1 h. The protein-Ni-NTA resin mixture was loaded and packed into a column. Contaminants were removed by washing the resin with washing buffer (8M urea, 0.1M NaH2PO4, 0.01M Tris-Cl, pH 6.3) and the fusion proteins were renatured using a linear 6M-1M urea gradient in 0.85% NaCl (pH 7.4) for 2h at each step. After renaturation, the proteins were eluted in 250mM imidazole and the eluates were concentrated and desalted using a (Vivascience, UK).

SDS-PAGE and Western blot of OVA-DT

The purity and identity of OVA-DT were examined by SDS-PAGE and Western blot.22 Protein separation was performed using 8% SDS-polyacrylamide minigels (Bio-Rad Laboratories Cambridge, MA) and 0.1% Coomassie blue (Sigma) for staining. For immuno-detection, proteins were transferred to a nitrocellulose membrane (Hybond-C; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) using the transfer apparatus supplied by Bio-Rad Laboratories, CA, USA. Immunoblots were blocked with 5% bovine serum albumin (BSA) in phosphate buffered saline containing 0.1% Tween 20 (PBST) for 1h and then incubated with a rabbit anti-OVA or a rabbit anti-DT serum at a dilution of 1:4000 in 5% BSA in PBST for 1h. After washing in PBST, the blots were incubated with HRP-conjugated goat anti-rabbit Ab diluted 1:6000 in 5% BSA in PBST for 1 h, developed with chemiluminescent reagents (SuperSignal; Pierce, Rockford, IL, USA) according to manufacturers instructions, and exposed to medical X-ray film (HR-G30; Fuji photo film Co., Tokyo).

Sensitization and active systemic anaphylactic shock

For OVA sensitization, BALB/c mice were injected once intraperitoneally with a mixture of OVA (100μg/mouse), aluminium hydroxide (20μg/mouse) and B. pertussis (1 x 10⁸ bacteria/mouse). ASAS was induced by intravenous injection with 100μg of OVA 15 days of the sensitization and measured using an ASAS scoring system (Table 1).23

Table 1. Scoring of Active Systemic Anaphylactic Shock

<table>
<thead>
<tr>
<th>Shock sore</th>
<th>Sign</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No sign</td>
</tr>
<tr>
<td>1</td>
<td>Decreasing bouts of spontaneous activity and piloerection</td>
</tr>
<tr>
<td>2</td>
<td>Loss of coordination and dyspnea</td>
</tr>
<tr>
<td>3</td>
<td>No activity following whisker stimuli and slight activity after prodding with ball-point pen</td>
</tr>
<tr>
<td>4</td>
<td>No activity following whisker stimuli, progressive paresis beginning with the behind leg</td>
</tr>
<tr>
<td>5</td>
<td>No activity following Hoffner's tail pinching stimuli by forcep</td>
</tr>
<tr>
<td>6</td>
<td>Brief but violent convulsion, prostration, coma or substantial recovery</td>
</tr>
<tr>
<td>7</td>
<td>Fatal shock (died within 30-60 min)</td>
</tr>
<tr>
<td>8</td>
<td>Fatal shock (died within 15 min)</td>
</tr>
</tbody>
</table>

Isolation of mast cells

Peritoneal exudate cells were collected from normal mice injected intraperitoneally with 10 ml of serum free RPMI 1640 medium with a syringe fitted with a no. 18-gauge needle, and contaminating red blood cells were lysed with an ACK lysing buffer.24 The peritoneal cells were then suspended in RPMI 1640 medium supplemented
with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 2.2 mM sodium bicarbonate, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY, USA; complete RPMI medium). Fifteen milliliters of the cell suspension (2 × 10⁶ cells/ml) were added to a 75 cm² plastic culture flask (Costar, Cambridge, MA, USA) and incubated for 3 h at 37°C in a humidified 5% CO₂ incubator. Non-adherent cells were harvested by gently shaking the culture flask. Mast cells from those cells were isolated by depleting Thy 1.2- and B220-positive cells with Dynabeads mouse pan T and pan B (Dynal AS, Oslo, Norway), respectively, and confirmed by staining with toluidine blue.

IgE-mediated binding of OVA-DT

One milliliter of the peritoneal mast cells (4 × 10⁶ cells) were incubated with 150 μl of heat-inactivated serum (harvested 15 days after sensitization) from Ov-mice or normal mice by shaking at 4°C for 30 min, and washing three times with PBS. The cells were suspended in 1 ml of PBS containing 10 μg of OVA-DT and placed on ice for 30 min; unbound OVA-DT was removed by washing three times with PBS. The cells were then resuspended in 1 ml of PBS, and 100 μl of heat-inactivated serum from rabbits immunized with diphtheria toxoid were added. Cells were then placed on ice for 30 min, washed three times with PBS, suspended in 200 μl of PBS, containing 2 μl of FITC-conjugated goat anti-rabbit immunoglobulins (IgM and IgG), placed on ice for a further 30 min, then rewarshed three times with PBS and observed under the fluorescent microscope.

Histamine assay

The peritoneal mast cells (4 × 10⁶ cells) were treated with serum from Ov-mice or normal mice and washed with PBS, as described in the 'IgE-mediated binding of OVA-DT' above. They were then suspended in 1 ml of a complete RPMI medium containing 10 μg of OVA-DT or OVA, incubated for 30 min at 37°C, and their culture supernatants harvested and stored at -20°C. The supernatant histamine concentration was then determined by automated fluorometry (Astoria TM analyzer series 300, Astoria Pacific International, USA).

Cytotoxicity by OVA-DT

To examine the cytotoxicity of OVA-DT, mast cells were treated with serum from Ov-mice and OVA-DT or OVA, as described in the 'histamine assay section' above, and then incubated for 6 h at 37°C. They were then washed twice with PBS, and their viability measured by staining with trypan blue and using a haemocytometer chamber.

Proliferation assay

The cytotoxic activity of OVA-DT to OVA-specific B cells was investigated by examining the proliferative responsiveness of splenic lymphocytes. Fifteen days after sensitization, the mice were intravenously treated with OVA-DT (200 μg/mouse) or saline, and two days later, the mice were killed and their spleens removed. The single cell suspensions of lymphocytes were prepared by gently teasing between two glass slides. Pooled cell suspensions were prepared from 3 mice per group. Red blood cells in the spleen cell suspension were lysed by ACK lysing buffer. The lymphocytes were adjusted to 1 × 10⁶ cells/ml in a complete RPMI medium and 200 μl of cell suspensions plated in 96-well round bottomed microtiter plates. The wells were added with OVA (100 μg/well), ConA (0.5 μg/well), or LPS (2.5 μg/well) and incubated at 37°C for 2 days (in case of ConA and LPS) or 4 days (in case of OVA). Cell proliferation was measured by determining 6 h⁻³H-thymidine (¹H-TdR, New England Nuclear Boston, MA, USA) incorporation.

Antibody assay

The levels of OVA-specific IgG subclasses in the serum were determined by ELISA using 96-well microtiter plates (Nunc, Copenhagen, Denmark) as follows: 21-100 μl/well of OVA (10 μg/ml) in PBS was added per well and left overnight at 4°C, the plates were then washed three times with PBST. The wells were blocked with 150 μl of PBS containing 10% BSA (PBS-BSA) for 2 h at RT, and
washed three times with PBST. The wells were then filled with 100 µl of the individual serum samples diluted with PBS-BSA and incubated at RT for 1 h and then washed five times with PBST. Biotin-conjugated rat anti-mouse IgG1, -mouse IgG2a, or -mouse IgG3 mAbs were then added to the wells at a concentration of 2 µg/ml, and the plates incubated at RT for 1 h then washed five times with PBST. 100 µl of 1/1,500 peroxidase-conjugated goat anti-biotin mAb was added to the wells, which were then incubated at RT for 1 h, washed five times with PBS-Tween, and reacted with peroxidase substrate (Sigma) at 37°C for 30 min. The reaction was stopped by adding dilute sulfuric acid, and read at an optical density (OD) of 405 nm using an ELISA plate reader. The OVA-specific IgE levels were measured by capture-ELISA, as previously described.21 Plates were coated with 100 µl/well of a purified anti-mouse IgE mAb (2 µg/ml) and blocked with 150 µl of PBS-BSA for 2 h at RT. The plates were then washed three times with PBST, and 100 µg/well of serum samples diluted with PBS-BSA were added and incubated for 2 h. 100 µl/well of the biotinylated OVA (10 µg/ml) prepared using an EZ-link Sulfo-NHS-LC-biotinylation kit (Pierce, Rockford, IL, USA) was added and incubated at RT for 1 h. The plates were then washed five times and 100 µl of 1/1,500 peroxidase-conjugated goat anti-biotin mAb was added/well. The plates were then incubated at RT for 1 h, washed five times with PBS-Tween, and reacted with peroxidase substrate (Sigma) at 37°C for 30 min. The reaction was stopped by adding dilute sulfuric acid, and read at an optical density (OD) of 405 nm using an ELISA plate reader. Standard curves for IgG subclasses and IgE were obtained using capture Abs (rat anti-mouse-IgG1, IgG2a, IgG3, and IgE) and purified mouse Abs (IgG1, IgG2a, IgG3, and IgE) (Pharmacia Fine Chemicals).

Cytokine assays

Os-mice were treated intravenously with OVA-DT or saline 15 days after sensitization, and after 2 days, their splenic lymphocytes were prepared as described in the proliferation assay method. For in vitro production of IFN-γ and IL-4, lymphocytes were adjusted to 1 x 10⁶ cells/ml in a complete RPMI medium and 1 ml of cell suspensions were plated in each well of 24-well tissue culture plates. OVA (100 µg/well) or ConA (2.5 µg/well) were added and the plates cultured for 2 days (in case of ConA) or 4 days (in case of OVA) at 37°C in a humidified 5% CO₂ incubator. The culture supernatants were then harvested from the culture plates and stored at -20°C. The concentration of IFN-γ and IL-4 in the supernatants was determined with murine IFN-γ and IL-4 ELISA kits (Endogen Inc., Boston, MA, USA).

Analysis of Results

All results are expressed as mean ± SD. Differences were analyzed by the Students t test. A p value of <0.05 was considered as statistically significant.

RESULTS

Expression and analysis of OVA-DT

OVA-DT was expressed in E. coli BL21 and purified using the Ni-NTA system. This fusion protein was composed of an OVA fragment containing the allergenic determinants (+484-+1,222) that induce hypersensitivity in mice, a DT fragment which contained A fragment (ADP-ribosylation catalytic domain, +1-+579) and transmembrane domain of B fragment (+580-+1,170). The binding domain of B fragment (+1,171-1,880) was removed in order to block the binding of DT to its cell receptors (Fig. 1). Purified OVA-DT was analyzed by SDS-PAGE (Fig. 2A) and by immunoblotting using rabbit anti-OVA or antidiaphtheria toxoid Abs (Fig. 2B); its molecular weight was approximately 75kDa.

The administration of OVA-DT did not cause any sign of allergic response in mice sensitized with OVA

Mice sensitized with a mixture of OVA (100 µg/mouse) and adjuvants 15 days previously and normal mice were used to evaluate the allergic and toxic effect of OVA-DT in the mice. The mice were challenged with OVA-DT (200 µg/mouse) or
A) SDS-PAGE

![SDS-PAGE Image]

B) Western blot

![Western Blot Images]

OV A (100μg/mouse) via tail veins to induce ASAS and comparatively evaluate the ASAS scores. When Os-mice were challenged with OVA-DT, all had zero ASAS scores. In contrast, Os-mice challenged with OVA died due to ASAS 15 to 30 min after OVA challenge and average ASAS scores were 7.4. In an investigation of the toxicity of OVA and OVA-DT, neither were found to cause any sign of acute toxicity when injected into normal mice (Table 2).

The time course of the protective effect of OVA-DT on the ASAS of OVA-sensitized mice

The time course of the protective effects of OVA-DT was examined by challenging the mice with OVA (100μg/mouse) at different time intervals after the injection of OVA-DT. Mice sensitized with a mixture of OVA and adjuvants 13 days earlier were treated with OVA-DT and challenged with OVA 15 min, 24 h, and 48 h after
Table 2. The Active Systemic Anaphylactic Shock Score in the OVA-sensitize Mice Challenged with OVA or OVA-DT

<table>
<thead>
<tr>
<th>Group</th>
<th>Challenged by</th>
<th>No. of mice</th>
<th>*No. of mice showing shock score</th>
<th>†Mean of shock score</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>OVA</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>OVA-DT</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Sensitized</td>
<td>OVA</td>
<td>10</td>
<td>6 4</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>OVA-DT</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

The mice were intraperitoneally sensitized with a single injection of mixture of OVA (100 μg/mouse) and adjuvants, aluminium hydroxide (20 μg/mouse) and B. pertussis (1 x 10⁹ bacteria/mouse). Induction of active systemic anaphylactic shock in the sensitized mice was performed by a single intravenous injection of OVA (100 μg/mouse) or OVA-DT (200 μg/mouse) on the 15th days after sensitization.  
*Shock score was determined as described in Table 1.  
†Mean of shock score (Shock score / No. of mice showing shock score) / No. of total mice tested.

Table 3. The Time Course of Therapeutic Effect of OVA-DT in Mice Sensitized with OVA

<table>
<thead>
<tr>
<th>OVA challenged after OVA-DT treatment</th>
<th>No. of mice</th>
<th>*No. of mice showing shock score</th>
<th>†Mean of shock score (mortality, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 1 2 3 4 5 6 7 8</td>
<td></td>
</tr>
<tr>
<td>10 min</td>
<td>10</td>
<td>7 3</td>
<td>7.3 (100)</td>
</tr>
<tr>
<td>24 hr</td>
<td>10</td>
<td>1 4 2 3</td>
<td>6.7 (30)</td>
</tr>
<tr>
<td>48 hr</td>
<td>10</td>
<td>2 3 5</td>
<td>2.3 (0)</td>
</tr>
</tbody>
</table>

The mice were intraperitoneally sensitized with a single injection of mixture of OVA (100 μg/mouse) and adjuvants, aluminium hydroxide (20 μg/mouse) and B. pertussis (1 x 10⁹ bacteria/mouse), and thirteen days later, the mice were treated with OVA-DT (200 μg/mouse) or saline. Induction of active systemic anaphylactic shock in the sensitized mice was performed by a single intravenous injection of OVA (100 μg/mouse) 15 min, 24 h, or 48 h after OVA-DT treatment. *Shock score was determined as described in Table 1.  
*Mean of shock score (Shock score / No. of mice showing shock score) / No. of total mice tested.

OVA-DT treatment, and their ASAS scores compared (Table 3). When Os-mice were treated with OVA-DT 15 min after challenge with OVA, all of the mice died from shock, seven mice after 30 min and three after 15 min. The average ASAS score for the mice was 6.7. However, when Os-mice were challenged with OVA 24 h after OVA-DT treatment, only three (3/10) died and the survivors (7/10) appeared to be seriously ill; the average ASAS score for the group was 6.7. In contrast, when mice were challenged with OVA 48 h after OVA-DT treatment, none of the mice (0/10) died and the average ASAS score for the group was 2.3, which reflected the signs of minor symptom. These results suggest that OVA-DT acted as an anti-shock agent that protected the Os-mice on OVA challenge due to ASAS, and indicates that the protective effects of OVA-DT can be expected after 48 h.

The effect of OVA-DT on the booster sensitization of mice sensitized with OVA

The therapeutic effect of OVA-DT in terms of unresponsiveness to OVA re-sensitization was examined. OVA challenge was began on 15 days after the initial sensitization. Two groups of OVA-DT treated-Os-mice were prepared. One group of mice received the initial treatment only in 13 days after the sensitization (single dose) and the others received two more treatments in two days interval (triple doses). In challenge experiments, none of the mice both groups were dead due to the OVA injected on the second day after the final treatments. Extent of therapeutic effect of OVA-DT was comparatively examined between two groups. The mice survived from the shock were re-sensitized with a mixture of OVA and adjuvants as booster to restore OVA allergic responses in them. In previous experiments, we noticed that

Table 4. Failure in Booster Sensitization of OVA Allergic Mice Treated with OVA-DT

<table>
<thead>
<tr>
<th>*OVA-DT</th>
<th>*Mean of shock score after OVA challenge (mortality, %)</th>
<th>^Booster</th>
<th>^Mean of shock score after OVA challenge (mortality, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated</td>
<td>7.4 (100)</td>
<td></td>
<td>6.3 (50)</td>
</tr>
<tr>
<td>Single treated</td>
<td>3.2 (0)</td>
<td>+</td>
<td>2.2 (0)</td>
</tr>
<tr>
<td>Triple treated</td>
<td>2.6 (0)</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* Mice sensitized with a mixture of OVA and adjuvants in 13 days earlier were divided into two groups. One group of mice was treated once with OVA-DT 13 days after the sensitization (single dose) and the other group was treated three times with OVA-DT in two days interval (triple doses).

* The mice were challenged i.v. with OVA on the second days after final treatment with OVA-DT and the therapeutic effect of OVA-DT was evaluated by means of active systemic anaphylactic shock score.

^Two days later, the mice survived in active systemic anaphylactic shock test were re-sensitized with the same mixture of OVA and adjuvants (+).

^The mice were re-challenged i.v. with OVA 15 days after re-sensitization and evaluated by means of active systemic anaphylactic shock score.

OVA-induced allergic responses in mice had been maintained only for a month (data not shown). The mice were then challenged with OVA 15 days after the booster sensitization. As a result, 50% of the mice received single dose (5/10) were survived, while 100% of those received triple doses were survived. These results indicated that triple doses rendered a complete protection (Table 4).

OVA-DT binds to mast cells via OVA-specific IgE

Mast cells express FcRI on their cell surfaces. Release of histamine along with other mediators by mast cells following binding of IgE and allergens with FcRI is known well to be the major mechanism of systemic anaphylactic shock. Our results demonstrated that OVA-DT did not cause OVA-induced ASAS in Os-mice. Accordingly, we investigated whether specific binding of OVA-DT is specific to mast cells bearing FcRI. Mast cells isolated from the peritoneal exudate of normal mice showed ~95% toluidine blue staining (Fig. 3A, × 100 and 3B, × 400). To determine the binding of IgE to FcRI, mast cells were incubated with serum from Os-mice or normal mice. The cells were washed and reacted with OVA-DT. The binding of OVA-DT was examined using anti-diphtheria toxoid Ab and FITC-conjugated goat anti-rabbit immunoglobulins (IgM & IgG). Stained cells were observed by light (Fig. 3C, × 400) and fluorescein microscopy (Fig. 3D, × 400). Specific immunofluorescence was detected only in the cells treated with serum from Os-mice (Fig. 3D) and not in those treated with serum from normal mice (data not shown). These results suggest that OVA-DT specifically binds to the mast cells via OVA-specific IgE bound FcRI on the cells.

The effect of OVA-DT on the production of histamine by mast cells.

The fact that OVA-DT prevented the active systemic anaphylactic response of Os-mice suggests the inhibition of histamine granule release by OVA-DT bound to the OVA-specific IgE expressed on the mast cells. Thus, two types of mouse peritoneal mast cells treated with OVA-immune serum or with normal serum were prepared to compare the cell responses to OVA-DT treatments. The amounts of histamine released from the mast cells treated with OVA-DT and with OVA-immune serum or with normal serum were measured. In brief, mouse peritoneal mast cells were incubated in serum from Os-mice at 4°C for 30 min. The cells were washed and cultured in media containing OVA-DT or OVA for 1 h at 37°C. The amount of histamine released in the media was measured by using automated fluorometry (Fig. 4). When the mast cells were treated with normal serum, the amounts of histamine release were not altered by the challenging antigens, OVA only (356 ± 34 ng/ml) or OVA-DT (345 ± 56 ng/ml). Whereas, when mast cells
treated with OVA-immune serum were challenged with OVA only, the amounts of histamine released from the cells was increased up to 592 ± 14 ng/ml (base line level was 344 ± 57 ng/ml). In contrast, the amounts of histamine released from the same mast cells were not enhanced by treatment with OVA-DT. The amount of histamine remained at base line level. This difference was statistically significant (p<0.05). The result suggests that OVA-DT binds specifically to mast cells bearing OVA-specific IgE and blocks the release of histamine granules into the media.

Cytotoxic effect of OVA-DT on OVA-specific IgE bearing mast cells

Specific mast cell death by OVA-DT was believed to be responsible for the decreased level of histamine in culture supernatants of the cells. Mast cells treated with OVA-immune serum and normal serum (for 30 min at 4°C) were incubated in culture media containing OVA-DT or OVA for 6 h at 37°C. The survival rates of the cells were measured by trypan blue assay (Fig. 5). Survival rates of normal peritoneal mast cells pretreated with normal serum were not affected by OVA or OVA-DT treatment. Viable cell counts were 13 ± 2 (×10⁶) cells/ml in OVA treated groups and 14 ± 1 (×10⁶) cells/ml in OVA-DT treated group. In contrast, survival rates of mast cell pretreated with serum from Os-mice were lower after OVA-DT treatment than after OVA treatment. Total viable cell numbers in groups treated with OVA and OVA-DT were 13 ± 4 (×10⁶) cells/ml.
Fig. 4. The effect of OVA-DT on the production of histamine by mast cells. Mast cells were obtained from the peritoneal exudate cells of normal mice. The exudate cells were pooled and cultured in a culture flask (75 cm²) for 3 h, and non-adherent cells were harvested from the culture flask. Mast cells of the non-adherent cells were isolated by depletion of Thy 1.2- and B220-positive cells with the Dynabeads mouse pan T and pan B, respectively, and confirmed by staining with toluidine blue. Mast cells (2×10⁶ cells) were reacted with the sera of mice sensitized or not sensitized with OVA at 4°C for 30 min and washed three times with PBS. The washed cells were cultured in the presence of OVA (10 μg) or OVA-DT (20 μg) for 1 h and their culture supernatants harvested. The level of histamine in the cultured supernatants was measured by automated fluorometry. Results indicated the means ± S.D. of three independent experiments. *p<0.05 vs cells treated with sensitized serum and OVA.

6 ± 1 (× 10⁷) cells/ml, respectively, and this difference was statistically significant (p<0.05).

The in vitro effect of OVA-DT on the proliferation of OVA-specific B cells of mice sensitized with OVA.

Since B cells are known to capture antigens via the low affinity IgE receptor (FcεRII) expressed on their surfaces,⁵ the effect of OVA-DT treatments on B cells was examined. The effect of treating Os-mice with OVA-DT or saline was studied by comparing the proliferative responses of splenic lymphocytes to T and B cell mitogens such as ConA and LPS (Table 5). In the case of ConA treated cells, enhancements in cell proliferation were noticed in both groups. However, cells post treated with OVA-DT to LPS responded to a lesser extent [6.2 ± 1.8 (x10⁶ cpm)]. The count was a half of that of the cells from the mice post treated with saline [12.2 ± 1.8 (x10⁶ cpm)] to the same level of LPS stimulation and this was statistically significant (p<0.05). Proliferative responses of the cells of both groups were examined to OVA, and the proliferation response of splenic lymphocytes from mice post treated with OVA-DT was found to be approximately a quarter [1.5 ± 0.8 (x10⁶ cpm)] of that of the saline treated mice [6.6 ± 0.9 (x10⁶ cpm)].

OVA-DT treated to OVA-sensitized mice increased the level of OVA-specific IgG2a

Changes in OVA-specific Abs production in Os-mice treated with OVA-DT were examined. For this exercise Os-mice were treated with OVA-DT (200 μg/mouse) or saline via tail veins once 15 days after sensitization. The mice were killed after a further two days and blood was collected from the vena cava. Serum Abs to OVA was assayed by ELISA. As shown in Table 6, OVA-DT treatment caused a slight reduction in serum IgE (968.4 ± 16.1 ng/ml) and IgG1 (223.0 ± 31.6 μg/ml) compared to that in mice treated with saline (OVA-specific IgE, 1,198.2 ± 20.5 ng/ml; OVA-specific IgG1, 319.1 ± 48.8 μg/ml) but this was not statistically significant. Whereas, levels of OVA-specific IgG2a increased in OVA-
Table 5. The Effect of in vivo Treatment with OVA-DT on the Proliferation of Splenic Lymphocytes from Mice Sensitized with OVA

<table>
<thead>
<tr>
<th>Treatment in vivo</th>
<th>³H-thymidine incorporation (×10³ cpm) by lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium only</td>
</tr>
<tr>
<td>Saline</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>OVA-DT</td>
<td>0.3 ± 0.0</td>
</tr>
</tbody>
</table>

The mice were intraperitoneally sensitized with a single injection of a mixture of OVA (300μg/mouse) and adjuvants, aluminium hydroxide (20μg/mouse) and B. pertussis (1×10⁷ bacteria/mouse), and thirteen days later, the mice were treated with OVA-DT (200μg/mouse) or saline. After 2 days, the mice were sacrificed and the splenic lymphocytes pooled from 3 mice of each group. Cells were seeded in 96-well round bottomed microtitre plates (2×10⁵ cells/200μl/well) and cultured in the absence or the presence of OVA (100μg/well), ConA (0.5μg/well), or LPS (25μg/well) for 2 days (in case of ConA and LPS) or 4 days (in case of OVA). Cell proliferation was determined by the technique of ³H-thymidine incorporation. Results shown are the mean ± S.D. of three independent experiments. *p<0.02 vs saline-treated group. †p<0.05 vs saline-treated group.

Table 6. The Effect of OVA-DT Treatment on the Production of OVA-specific Antibodies in OVA Sensitized Mice

<table>
<thead>
<tr>
<th>Treatment in vivo</th>
<th>Level of OVA-specific immunoglobulin isotypes in serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG1 (g/ml)</td>
</tr>
<tr>
<td>Saline</td>
<td>319.1 ± 48.8</td>
</tr>
<tr>
<td>OVA-DT</td>
<td>223.0 ± 31.6</td>
</tr>
</tbody>
</table>

OVA-sensitized mice were intravenously treated with OVA-DT (200μg/mouse) or saline 13 days after sensitization, and thirteen days later, they were treated with OVA-DT (200μg/mouse) or saline. The mice were sacrificed 48h after the OVA-DT treatment and blood from 3 mice from each group was harvested. The levels of OVA-specific immunoglobulin isotypes were determined by ELISA as described in Materials and Methods. Each value indicated mean ± S.D. of three independent experiments. *p<0.05 vs saline-treated group.

DT treated mice (65.1 ± 5.4μg/ml) compared to the control mice (43.9 ± 3.6μg/ml). However, differences in the levels of OVA-specific IgG3 were not observed.

OVA-DT treatment in OVA-sensitized mice increased the production of IFN-γ by splenic lymphocytes but decreased IL-4

Changes in the production of IFN-γ and IL-4 by splenic lymphocytes from Os-mice treated with OVA-DT were investigated. Os-mice were treated with OVA-DT (200μg/mouse) or saline via tail veins once 15 days after sensitization. Two days later, splenic lymphocytes were isolated and cultured in media with ConA or OVA for 2-5 days. IFN-γ and IL-4 levels in the culture supernatants were assayed by ELISA. As shown in Table 7, after ConA stimulation, production of IFN-γ (3.479.6 ± 523.9 pg/ml) by lymphocytes from OVA-DT treated mice was higher than of the cells from saline treated mice (2.364.5 ± 235.3 pg/ml). Although these observations were reproduced by cells stimulated with OVA instead of ConA, the differences between the extent of IFN-γ production by cells from OVA-DT treated mice and that from the saline controls were more exaggerated and this was also statistically significant (p<0.01). In contrast, in experiments using IL-4, the results were the reverse of those observed for ConA stimulated cells, and a lower level of IFN-γ production by the cells from OVA-DT treated mice (18.2 ± 5.9 pg/ml) was observed compared to the saline treated mice 45.0 ± 5.8 pg/ml, which was also statistically significant (p<0.05).

DISCUSSION

A fusion protein was constructed by combining the allergen OVA with DT as a means of eliminating cells responsible for allergy reactions such as mast and B cells. The hypothesis was...
based on the following ideas, 1) the fusion protein, OVA-DT, should bind to cells only through OVA-specific surface IgE or its IgE bound receptors, FcRI or FcRII, 2) the immune complex of IgE and OVA-DT including the toxin would then be transported into the cytoplasm and that DT should result in the specific death of FcRI or FcRII bearing mast cells and other similar cells involved in the allergy reaction. The specificity of the DT induced cytotoxicity should be solely depending upon the OVA complex with specific IgE as the normal receptor-binding domain of DT, which might have caused non-specific toxicity was removed. Internalization of an IgE-imunonotoxin into the cytoplasm of cells, especially B cells has previously been reported.27,28

An animal model of active systemic anaphylactic shock to OVA was established using BALB/c mice.25 This system proved an ideal model for this allergy study. Throughout the experiments, the routes of inoculation of OVA into the mice were fixed, as intraperitoneal for the sensitization and intravenous for the challenges. After a single dose of OVA sensitization, the mice became an ideal model, the Os-mice, which expressed ASAS to OVA challenge on every occasion. Fifteen days after sensitization, typical ASASs were reproduced by OVA challenges. This fatal anaphylactic response induced by the initial sensitization was demonstrated over a period of 30 days. The fusion protein, OVA-DT, was confirmed to be nontoxic in normal mice, unless an extreme dose (> 800 μg/mouse) was introduced (data not shown). OVA-DT, was inoculated into the Os-mice via tail veins thirteenth days after sensitization, and a remarkable therapeutic effect of OVA-DT was then observed in the Os-mice treated with OVA-DT (200 μg/mouse). The protective effect of OVA-DT in the mice from ASAS was demonstrated as early as 24 h after the treatment, and complete cure was observed 48 h after treatment. This effect of OVA-DT in mice was examined with respect to their responses to OVA re-sensitization. Surprisingly, mice that received three times with OVA-DT remained unresponsive to OVA challenges. These results suggested that a single dose of OVA-DT is not enough to make the mice free from OVA shock.

In this respect, mast cells were considered to be the primary target for the rapid and complete protection offered by OVA-DT. It is generally accepted in theory that a rapid release of stored mediators, notably histamine, from the high affinity IgE receptor (FcRII) bearing mast cells and other associated cells, such as eosinophils,29,30 basophils,31,32 and Langerhans cells,33,34 following exposure to allergen is the most important process involved in the allergy reaction.35,36

The specific binding of OVA-DT on mast cells was firstly examined. By immunofluorescent microscopy, specific binding of OVA-DT was observed only on those mast cells treated with serum from Os-mice (Fig. 3D). These results indicated that OVA-DT binding on mast cells was mediated by OVA specific IgE in the serum from Os-mice, which were bound to FcRII on the mast cells. A significant reduction in histamine release by OVA-DT treatment was recorded in mast cells treated with serum from Os-mice compared to that expressed by cells treated with normal serum. The reduction of mast cells due to OVA-DT containing cytotoxic DT was considered as the

major effect especially in vivo. Decrease in the absolute number of mast cells was indirectly evidenced by confirming the cytotoxic effect of OVA-DT on mast cells in vitro. Thus, it was tentatively concluded that OVA-DT blocks the deregulation of histamine from mast cells and eventually and specifically induces the death of the mast cells.

Lymphocytes from spleens were prepared from Ov-mice treated with OVA-DT or saline. A relative number of specific cell types in the population were compared in terms of their proliferative responses to mitogens or OVA antigen. The stimulants compared were, ConA for T cells, LPS for B cells, and OVA for specific cells. Thus, the effect of OVA-DT on T cells was believed to be minor or insignificant based upon the fact that the proliferation rates of the cells stimulated by ConA did not vary in the mice treated with saline or OVA-DT. By contrast, in experiments with LPS, a significant decrease in proliferation was observed in lymphocytes from the OVA-DT treated mice, which strongly suggests that B cells were the targets of the cytotoxin. By stimulation with the specific antigen OVA, further exaggerated decreases in the proliferation rates of lymphocytes were observed in the treated mice, which indicated strongly that OVA primed B cells were the specific targets of OVA-DT.

These OVA primed B cell group might include not only B cells bearing IgE but also B cells expressing IgG. If OVA-DT destroys B cells bearing OVA specific IgE and/or FcRN, changes of IgG-producing B cell population and IgG production in the treated mice seemed likely. In the serum of OVA-DT treated mice, decrease in the levels of both IgE and IgG1 were not significant but IgG2a increased significantly increased. Thus, an effect of the OVA-DT treatment was to eliminate IgE bearing cells and cause the B cell to produce switched OVA specific Ig isotype from IgM to IgG2a. In this respect, IL-4 and IFN-γ known to be the switching factors for IgG1/IgE and IgG2a, respectively, were examined. Surprisingly, increased IFN-γ production was observed in lymphocytes from OVA-DT treated mice. Since T cells are known to produce these cytokines, the further enhancement of cytokines by T cell mitogens was examined. As expected, further enhanced by the same lymphocytes was observed by stimulating them with ConA. A much higher level of enhancement was noticed when OVA were used as stimulant. In contrast, the production of IL-4 by the lymphocytes of the treated mice decreased. These results suggest that OVA-DT changed the cytokine profile of T cell response from Th2 to Th1 in OVA-sensitized mice.

In conclusion, our results demonstrate a new therapeutic approach, based on the use of that specifically eliminates allergen specific mast cells. Furthermore, OVA-DT treatment induced a switching of the immune response from Th2 to Th1 by increasing the level of IFN-γ. OVA-DT could provide an immediate therapy for allergy and offers a complete cure to animals when treated over three times.

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