

Allergen-specific immunosuppression by ovalbumin fused with diphtheria toxin in mice sensitized with albumins of different origin

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Summary

Background We previously reported that ovalbumin–diphtheria toxin (OVA–DT) fusion protein eliminates mast cells bearing OVA-specific IgE and protects OVA-sensitized mice from fatal anaphylaxis induced by OVA challenge.

Objective To prove the specificity of therapeutic effect of OVA–DT to allergy induced by OVA only and not by other allergens such as human serum albumin (HSA), and to examine the cytotoxic effect of OVA–DT on B cells bearing OVA-specific IgE.

Methods Mice were sensitized with two different antigens, OVA and HSA, and then treated with OVA–DT. The therapeutic effect of OVA–DT on the allergy response to each of allergen was evaluated by anaphylactic test. The effect of OVA–DT on the production of allergen-specific Ig isotypes of the sensitized mice and the cytotoxic effect of OVA–DT on B cells expressing OVA-specific IgE were examined.

Results OVA–DT suppressed only OVA-induced allergy but not HSA-induced allergy in mice sensitized with a mixture of OVA and HSA. The suppression was prolonged even to the mice boosted with the same allergen 14 days after last treatment of OVA–DT.

In addition, when the sensitized mice were boosted with the same allergens 14 days after last treatment of OVA–DT, the mice showed to increase the production of OVA-specific IgG2a/IgG3 and decreased that of OVA-specific IgE. OVA–DT targeted B cells bearing OVA-specific IgE, and killed them by DT-mediated cytotoxicity.

Conclusion The therapeutic effect of OVA–DT was specific to OVA-induced allergy and the suppression of OVA-induced allergy was continuously shown in the mice boosted with the same allergens. This is considered to be caused by the increase of OVA-specific IgG2a and IgG3, and because of the decrease of OVA-specific IgE by killing of B cells bearing OVA-specific IgE.

Keywords allergen–toxin fusion protein, diphtheria toxin, immunotherapy, ovalbumin

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Introduction

For an ideal immunotherapy of allergy, several novel approaches have been attempted to develop modified [1–4] or recombinant allergens [5–8] that are capable of reducing the anaphylactic shock and saving beneficial immunogenicity. Some researchers have made efforts to construct the chemical conjugates of allergens with non-toxic B subunit of *Escherichia coli* [9] or cholera toxin [10–12], anti-IgE with toxic A subunit of ricin [13, 14], or to construct the recombinant fusion proteins of cytokine and allergen [15]. The aims of these works are to induce immune tolerance to allergen, to provide the cytotoxic agent to cell surface

molecules that will be internalized and kill the cells, or to induce the shifting of T cell response from T helper (Th)2 to Th1. However, the application of such products in clinic is at an early stage and the therapeutic efficacies of the products are still in debate.

In a previous study, to eliminate mast cells, we constructed ovalbumin–diphtheria toxin (OVA–DT). It was demonstrated that OVA–DT protects OVA-sensitized mice from fatal anaphylactic shock caused by OVA challenge. And also, it was found that OVA–DT in the presence of mouse anti-OVA serum binds to mast cells, and thereby kills them [16].

In this study, to prove the specificity of therapeutic effect of OVA–DT on OVA-induced allergy, we sensitized mice with a mixture of albumins from different origins, OVA and human serum albumin (HSA). And we further examined the cytotoxic effect of OVA–DT on B cells bearing OVA-specific IgE on their surfaces, and the modulatory effect of OVA–DT on the OVA-specific Ig isotypes.

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Materials and methods

Mice

Female BALB/c mice (6–7 weeks of age) bred under specific pathogen-free conditions were used for studies. These mice were obtained from the Korean Institute of Science and Technology (Daejeon, Korea) and maintained in a pathogen-free animal facility at Yonsei University College of Medicine (Seoul, Korea).

Allergens, mitogens and adjuvants

OVA and HSA used as allergens for sensitization of mice, and lipopolysaccharide (LPS) used as B cell mitogens were purchased from Sigma Chemical Co. (St Louis, MO, USA). Formalin-inactivated *Bordetella pertussis* and aluminium hydroxide used as adjuvants were purchased from Green-Cross Co. (Seoul, Korea) and Aldrich Chemical Co. (Milwaukee, WI, USA), respectively.

Antibodies

Purified mouse IgE and IgG subclasses (IgG1, IgG2a and IgG3), rat anti-mouse IgE monoclonal antibody (mAb), biotin-conjugated rat anti-mouse IgE and rat anti-mouse IgG subclasses mAbs were purchased from PharMingen (San Diego, CA, USA). Peroxidase-conjugated goat anti-biotin Ab and tetramethylbenzidine were purchased from Sigma and PharMingen, respectively. Anti-diphtheria toxoid Ab was prepared from rabbit immunized with diphtheria toxoid once a week for 4 weeks. Anti-OVA Abs were prepared both from rabbit and mouse immunized with a mixture of OVA and complete or incomplete Freund's adjuvant once a week for 4 weeks. Anti-HSA Ab was prepared from mouse immunized with a mixture of HSA and complete or incomplete Freund's adjuvant once a week for 4 weeks. Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgE heavy-chain Ab was purchased from Serotec (Oxford, UK).

OVA–DT

OVA–DT was prepared as described previously [16]. This fusion protein consisted of two kinds of protein, an OVA fragment contained the allergenic determinants capable of

inducing allergy in mice and a DT fragment which contained A fragment (adenosine diphosphate-ribosylation catalytic domain) and transmembrane domain of B fragment. The receptor-binding domain of B fragment was removed in order to block binding of DT to its receptor. The purity and identity of OVA–DT were examined by SDS-PAGE and Western blot.

Sensitization, immunotherapy, boosting and anaphylaxis

All procedures for injection was carried out at day indicated in Fig. 1. Sensitization of mouse was performed by means of intraperitoneal (i.p.) injection once with a mixture of OVA (100 µg/mouse), HSA (100 µg/mouse), aluminium hydroxide (20 µg/mouse) and formalin-inactivated *B. pertussis* (1×10^9 bacteria/mouse). The sensitized mice were referred to as OVA/HSA-sensitized mice. The treatment was intravenously (i.v.) injected with 100 µg of OVA–DT once a day for 3 days. The challenge for induction of anaphylaxis was performed by means of intravenous injection with 100 µg of OVA or HSA. The boosting of OVA/HSA-sensitized mice was performed by means of i.p. injection once with 100 µg of OVA, and the mice were referred to OVA/HSA-boosted mice.

Anaphylactic reactions were graded at different time-points according to the following scoring system: 0, no sign of reaction; 1, scratching and rubbing around the nose and head; 2, decreased activity with an increasing respiratory rate, pilar erecti and/or puffing around the eyes; 3, laboured respiration and cyanosis around the mouth and tail; 4, slight or no activity after prodding or tremors and convulsion; 5, death [17].

Antibody assay

Blood was harvested through vena cava of mouse, and sera were stored at -20°C until assay. The concentrations of OVA- or HSA-specific IgG subclasses in the serum were determined by ELISA as described in previous study [16]. One hundred microlitres (20 µg/mL) of OVA or HSA was coated on ELISA plates (NUNC, Roskilde, Denmark), and sera from mice were used as test samples. Primary antibodies used were biotin-conjugated rat anti-mouse IgG1, IgG2a and IgG3, and secondary antibody was peroxidase-conjugated goat anti-biotin Ab. The concentrations of OVA- or HSA-specific IgE were measured by capture ELISA [18]. The plates

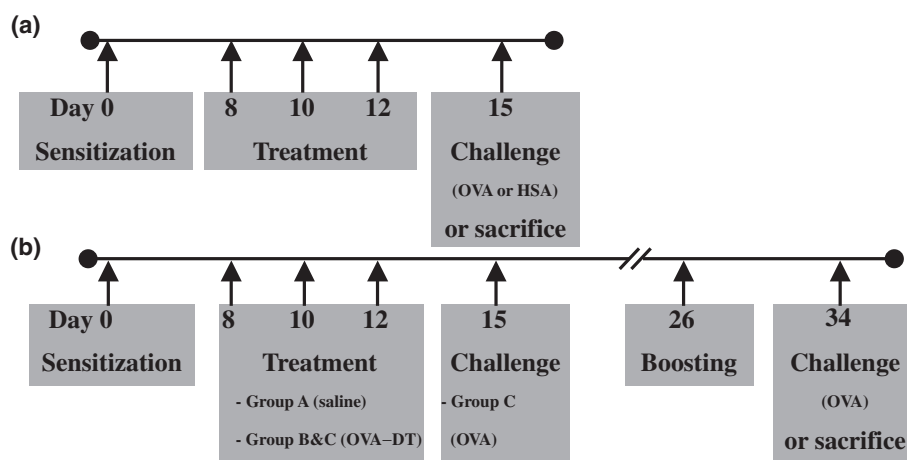


Fig. 1. Protocols used for mouse sensitization and immunotherapy with ovalbumin–diphtheria toxin (OVA–DT): (a) Mice were sensitized with a mixture with OVA, human serum albumin (HSA) and adjuvants at day 0, and treatment of OVA–DT or saline was performed at days 8, 10 and 12. At day 15, the mice were challenged with OVA or HSA, or killed. (b) OVA/HSA-sensitized mice were divided into three groups. Group A was treated with saline, and groups B and C were treated with OVA–DT. The treatment was performed once a day at days 8, 10 and 12. Only group C was challenged with OVA at day 15. All three groups were boosted with the same allergens used in primary sensitization at day 26. The mice were challenged with OVA or killed at day 34.

were coated with a purified rat anti-mouse IgE, and sera from mice were used as test samples. The biotinylated OVA or HSA prepared using an EZ-LINK sulphy-NHS-LC-biotinylation kit (Pierce, Rockford, IL, USA) were used, and peroxidase-conjugated goat anti-biotin Ab was used as secondary antibody. The reactions were read with an ELISA plate reader at 450 nm. The known mouse Ig isotypes, IgG1, IgG2a, IgG3 and IgE, were used for calibrating the assay.

Preparation of IgE-expressing B cells

Lymphocytes were isolated from OVA/HSA-sensitized mice killed 15 days after sensitization, and red blood cells contaminated were lysed by ammonium-chloride-potassium (K) (chloride) lysing buffer [19]. The cells (9×10^7 cells) were suspended in 25 mL of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 2.2 mM sodium bicarbonate, 100 U/mL penicillin and 100 µg/mL streptomycin (GICO BRL, Grand Island, NY, USA) (hereafter, referred to as the complete medium), and cultured in 75 cm² plastic culture flask (Corning Inc., NY, USA) for 3 h at 37 °C in a humidified 5% CO₂ incubator (hereafter, referred to as the incubator). Non-adherent cells were harvested by gently shaking the culture flask. B cells from non-adherent cells were isolated by depleting Thy 1.2-positive cells with Dynabeads mouse pan T (Dynal AS, Oslo, Norway). The cells (4×10^7 cells/mL) were stained with 5 µg of FITC-conjugated rat anti-mouse IgE heavy chain at 4 °C for 1 h and washed three times with phosphate-buffered saline (PBS)–1% FCS. The isolated B cells were sorted by using fluorescence-activated cell sorter (FACS; Beckman Coulter, Miami, FL, USA). The sorting process was repeated several times to obtain a lot of B cell expressing IgE. More than 92% of the cells sorted were found out as IgE-expressing B cells by FACS analysis.

Cytotoxicity of OVA–DT

IgE-expressing B cells isolated from OVA/HSA-sensitized mice were adjusted to 5×10^6 cells in 10 mL of the complete medium supplemented with 10 µg/mL of LPS, and cultured in 25 cm² plastic culture flask (Corning Inc.) for 24 h in incubator. The cells were harvested, washed three times with PBS–1% FCS, suspended in 2 mL of complete medium, and divided into four groups (1×10^6 cells/500 µL/group): One was treated with a mixture OVA–DT (20 µg) and serum (50 µL) from OVA-sensitized mouse (anti-OVA serum),

another treated with a mixture of OVA–DT (20 µg) and serum (50 µL) from HSA-sensitized mouse (anti-HSA serum), another treated with a mixture of OVA–DT (20 µg) and serum (50 µL) from non-sensitized mouse (non-immune serum) and the other treated with medium only. The cells were incubated in 1.5 mL Eppendorf tube (Eppendorf AG, Hamburg, Germany) for 3 h and washed three times with PBS–1% FCS. The cells were stained with trypan blue dye and the survived cells were counted by haemocytometer chamber.

Analysis of data

All data were expressed as the mean $\pm$ SD. The differences were analysed using the Student's *t*-test. A *P*-value of <0.05 was considered as statistically significant.

Results

The specificity of immunotherapy by OVA–DT

To prove that OVA–DT suppresses specifically only the OVA-induced allergy without interfering immune response to other antigen, the procedures were performed as shown in Fig. 1a. OVA/HSA-sensitized mice were treated with OVA–DT or saline, and challenged with OVA or HSA. Not surprisingly when saline-treated group was challenged with OVA or HSA, within 1 h, they all died from OVA-induced anaphylaxis (symptom score, 5.0) or HSA-induced anaphylaxis (symptom score, 5.0), respectively. And, when OVA–DT-treated group were challenged with HSA, they also died by HSA-induced anaphylaxis (symptom score, 5.0). In contrast, not one mouse from the OVA–DT-treated group died when challenged with OVA, and showed only signs of minor symptom (score, 1.6) (Table 1). These results suggest that therapeutic effect of OVA–DT is specific to OVA-induced allergy.

Duration of suppression of OVA-induced allergy

To find out the duration of suppression of OVA-induced allergy by OVA–DT, OVA/HSA-sensitized mice were treated with OVA–DT or saline, boosted with the same allergens, and challenged with OVA as shown in Fig. 1b and in Materials and methods.

In saline-treated group A, all mice died within 1 h from OVA-induced anaphylaxis, and the symptom score was 5.0.

Table 1. Symptom score of anaphylaxis following OVA or HSA challenge in OVA/HSA-sensitized mice treated or not treated with OVA–DT

Treatment after sensitization	Challenge	Number of mice	Number of mice showing symptom score					*Mean of shock score	
			0	1	2	3	4		5
Saline	OVA	7						7	5
	HSA	7						7	5
OVA-DT	OVA	10	2	3	4	1			1.6
	HSA	10						10	5

Protocol was described in Fig. 1a. The mice were sensitized with a mixture of OVA, HSA and adjuvants, and injected i.v. with OVA–DT or saline once a day for 3 days. For the induction of anaphylaxis, the mice were challenged i.v. with OVA or HSA. Symptom score of anaphylaxis was determined as described in Materials and methods.

*Mean symptom score = symptom score $\times$ number of mice showing the symptom score/number of total mice tested.

OVA, ovalbumin; HSA, human serum albumin; DT, diphtheria toxin; i.v., intravenously.

Table 2. An extended therapeutic effect of OVA–DT on the anaphylaxis of OVA/HSA-sensitized mice boosted with the mixture of allergens used in primary sensitization

Group	Number of mice	Number of mice showing symptom score						*Mean of shock score
		0	1	2	3	4	5	
A	10						10	5
B	12	1	4	5	2			1.7
C	8	3	3	2				0.6

Protocol was described in Fig. 1b. OVA/HSA-sensitized mice were divided into three groups. Group A was treated with saline, and groups B and C were treated with OVA–DT. Group C was challenged with OVA. All groups were boosted with the same antigens used in the primary sensitization, and challenged i.v. with OVA.

OVA, ovalbumin; HSA, human serum albumin; DT, diphtheria toxin; i.v., intravenously.

*Mean symptom score = (symptom score × number of mice showing the symptom score)/number of total mice tested.

However, not one mouse in OVA–DT-treated group B died from OVA-induced anaphylaxis and showed low symptom score (1.7). Also, mice from group C, challenged (day 15) and re-challenged (day 34) with OVA, did not die and the symptom score was 0.6 (Table 2). Taken together, although the OVA/HSA-sensitized mice, which were challenged or not challenged with OVA 3 days after last treatment of OVA–DT, were boosted with the same allergens, the mice did not die from OVA-induced anaphylactic shock. This suggests that the suppressions of OVA-induced allergy shown in OVA/HSA-sensitized mice treated with OVA–DT were extended even to the mice boosted with the same allergen.

The effect of OVA–DT on the production of OVA- and HSA-specific Ig's in OVA/HSA-sensitized mice and OVA/HSA-boosted mice

To obtain antibodies, OVA/HSA-sensitized mice were killed at day 15 (Fig. 1a) and OVA/HSA-boosted mice at day 34 (Fig. 1b). As for the OVA-specific Ig isotypes in OVA/HSA-sensitized mice treated with OVA or saline, the result is shown in Fig. 2a. Only the concentration of OVA-specific IgG1 showed no difference between OVA–DT-treated group ($38\,818 \pm 969$ ng/mL) and saline-treated group ($39\,177 \pm 662$ ng/mL). The concentrations of other three isotypes showed differences between the groups. The concentrations of OVA-specific IgG2a and IgG3 were markedly increased in OVA–DT-treated group ($10\,065 \pm 214$ and 7327 ± 460 ng/mL, respectively) compared with those of saline-treated group (5992 ± 284 and 3868 ± 159 ng/mL, respectively). The differences in the concentrations of OVA-specific IgG2a and IgG3 between OVA–DT-treated group and saline-treated group were statistically significant. In reverse to the statistics for OVA-specific IgG2a and IgG3, the concentration of OVA-specific IgE was lower in the OVA–DT-treated group (272 ± 22 ng/mL) than in saline-treated group (612 ± 46 ng/mL), and this was statistically significant.

In case of HSA-specific Ig isotypes in OVA/HSA-sensitized mice treated with OVA or saline (Fig. 2b), OVA–DT did not affect the production of HSA-specific Ig isotypes of

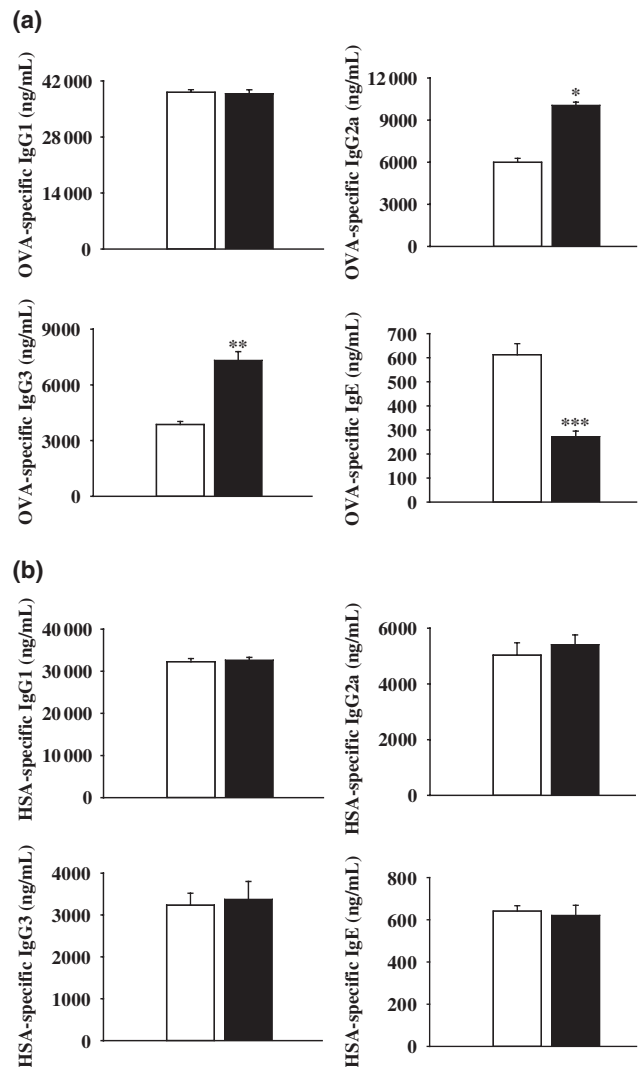


Fig. 2. Comparison of the concentrations of ovalbumin (OVA)- and human serum albumin (HSA)-specific Ig isotypes between the sera of OVA/HSA-sensitized mice treated and not treated with OVA–diphtheria toxin (OVA–DT). OVA/HSA-sensitized mice were treated intravenously with saline (□) or OVA–DT (■), and killed as shown in Fig. 1a. Sera were obtained from three mice in each group and pooled. The concentrations of the Ig isotypes in serum were determined by ELISA. Each value represents the means $\pm$ SD of three independent experiments. * $P = 0.005$ vs. IgG2a of saline-treated group, ** $P = 0.005$ vs. IgG2a of saline-treated group, *** $P = 0.001$ vs. IgE of saline-treated group.

OVA/HSA-sensitized mice. The concentrations of HSA-specific IgG1, IgG2a, IgG3 and IgE in OVA–DT-treated group were $32\,683 \pm 606$, 5409 ± 349 , 3377 ± 421 and 621 ± 47 ng/mL, respectively, and those of saline-treated group were $32\,264 \pm 752$, 5033 ± 442 , 3235 ± 284 and 641 ± 25 ng/mL, respectively. These results show that treatment of OVA–DT to OVA/HSA-sensitized mice increased the production of OVA-specific IgG2a and IgG3 and decreased that of OVA-specific IgE, but did not affect that of HSA-specific Ig isotypes.

The concentrations of OVA-specific Ig isotypes in OVA/HSA-boosted mice were examined (Fig. 1b). The pattern of the concentrations of Ig isotypes of OVA/HSA-boosted mice killed 22 days after the last treatment of OVA–DT or saline (Figs 1b and 3) was similar to that of OVA/HSA-sensitized

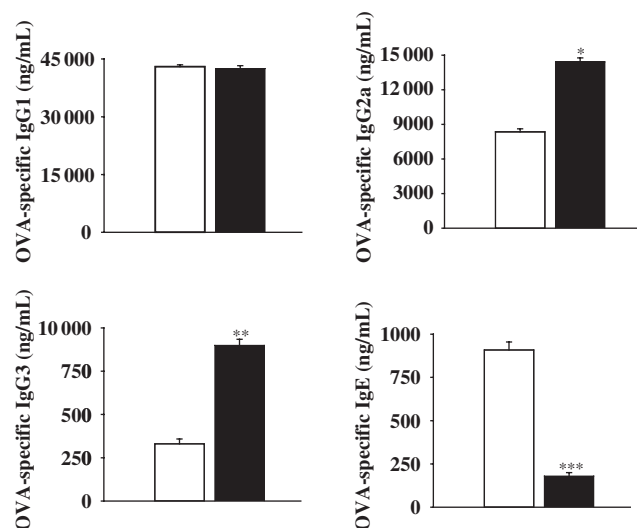


Fig. 3. Comparison of the concentrations of ovalbumin (OVA)-specific Ig isotypes between the sera of OVA/human serum albumin (HSA)-boosted mice treated with OVA-diphtheria toxin (OVA-DT) and saline. OVA/HSA-sensitized mice were boosted with the same allergens 14 days after the last treatment of saline (□) or OVA-DT (■). Eight days later, the mice were killed, and sera were obtained from three mice in each group and pooled. Each value represents the means $\pm$ SD of three independent experiments. * $P = 0.002$ vs. IgG2a of the saline-treated group. ** $P = 0.002$ vs. IgG3 of saline-treated group. *** $P = 0.002$ vs. IgE of saline-treated group.

mice killed 3 days after last treatment (Figs 1a and 2a). The concentrations of OVA-specific IgG1 obtained from OVA/HSA-boosted mice showed insignificant little difference between OVA-DT-treated group and saline-treated group. However, the concentrations of both OVA-specific IgG2a and IgG3 in OVA-DT-treated group (14422 ± 343 and 8996 ± 347 ng/mL, respectively) were also higher than those of saline-treated group (8355 ± 262 and 3300 ± 286 ng/mL, respectively). The differences of the concentrations of OVA-specific IgG2a and IgG3 between OVA-DT-treated group and saline-treated group appeared to be statistically significant, respectively. In reverse to the statistics for OVA-specific IgG2a and IgG3, the concentration of OVA-specific IgE in OVA/HSA-boosted mice was lower in OVA-DT-treated group (220 ± 19 ng/mL) than saline-treated group (908 ± 46 ng/mL), and the difference was statistically significant.

The cytotoxic effect of OVA-DT on B cells expressing OVA-specific IgE

To clarify the reason for the decrease of concentration of OVA-specific IgE in OVA/HSA-sensitized mice treated with OVA-DT, we isolated IgE-expressing B cells from OVA/HSA-sensitized mice, and examined the cytotoxic effect of OVA-DT on the cells as described in Materials and methods. As shown in Fig. 4, the survival rate of the cells treated with OVA-DT in the presence of non-immune serum ($82 \pm 8.1\%$) was slightly decreased compared with that of cells treated with medium only ($90.4 \pm 3.8\%$), but the difference was statistically insignificant. The survival rate of the cells treated with OVA-DT in the presence of anti-HSA serum ($80.4 \pm 9.8\%$) was also shown to be similar to that of cells treated with OVA-DT in the presence of non-immune serum. In contrast, the survival rate of the cells treated with OVA-DT in the presence of anti-OVA serum ($58.1 \pm 6.2\%$) appeared to

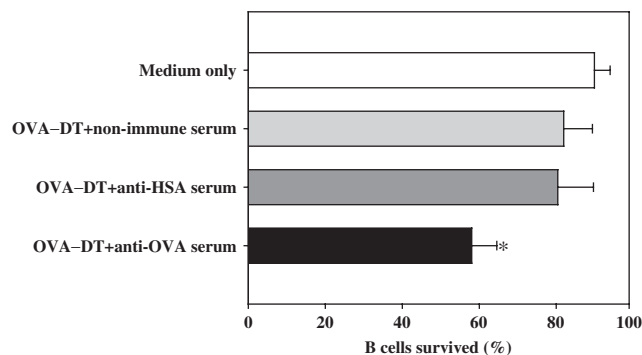


Fig. 4. The cytotoxic effect of ovalbumin-diphtheria toxin (OVA-DT) on IgE-bearing B cells from OVA/human serum albumin (HSA)-sensitized mice. The isolated IgE-expressing B cells stimulated with lipopolysaccharide for 24 h were divided into four groups: One was treated with a mixture of OVA-DT and anti-OVA, the second with a mixture of OVA-DT and anti-HSA, the third with OVA-DT and non-immune serum, and the fourth with medium only. The cells were incubated for 3 h, and washed with phosphate-buffered saline-1% fetal calf serum. The survived cells were counted by trypan blue dye exclusion, and expressed as survival rate (%). Each value represents the means $\pm$ SD of three independent experiments. * $P = 0.003$ vs. OVA-DT and non-immune serum.

be markedly reduced compared with that of cells treated with OVA-DT in the presence of non-immune serum, and the difference was statistically significant. These results show that OVA-DT kills IgE-expressing B cells from OVA/HSA-sensitized mice in the presence of anti-OVA but not anti-HSA, suggesting that the cytotoxicity of OVA-DT is specific for B cells expressing OVA-specific IgE.

Discussion

This study demonstrated that OVA-DT protects OVA/HSA-sensitized mice from OVA-induced anaphylaxis but not from HSA-induced anaphylaxis. This proves that immunotherapy of OVA/HSA-sensitized mice by OVA-DT is specific to OVA-induced allergy without interfering immune response to HSA.

Previously, we reported that OVA-DT binds and destroys the mast cells that bear OVA-specific IgE [16]. However, restoration of the allergic response to OVA is assumable because reconstitution of mast cells from haematopoietic systems into the circulation was to be expected.

In our study, although the OVA/HSA-sensitized mice treated with OVA-DT were boosted with the same allergens as shown in Table 2, OVA-induced allergy in the mice was still shown to be suppressed. This suggests that OVA-DT therapy involves possible additional events to the direct deletion of OVA-specific mast cells.

These assumptions are supported by the outcome of concentrations of OVA-specific Ig isotypes of OVA/HSA-sensitized mice killed 3 days after the last treatment of OVA-DT (Fig. 1a) and those of the boosted mice killed at 34 days (Fig. 1b). OVA-DT in OVA/HSA-sensitized mice increased the production of OVA-specific IgG2a and IgG3, and decreased that of OVA-specific IgE. On the other hand, OVA-DT in OVA/HSA-sensitized mice was shown not to affect the productions of HSA-specific IgG2a, IgG3 and IgE. These support that in OVA/HSA-sensitized mice, OVA-DT

specifically modulates the productions of OVA-specific Ig isotypes but not the production of HSA-specific ones.

Similar pattern of increases and decrease in the production of OVA-specific IgG2a, IgG3 and OVA-specific IgE were also detected in OVA/HSA-boosted mice. From the pattern humoral immune response of OVA/HSA-sensitized mice modulated by OVA–DT extended to OVA/HSA-boosted mice, we can infer that the modulation of the productions of OVA-specific Ig isotypes in OVA/HSA-sensitized mice may be a part of additional events of OVA–DT.

A production of the blocking antibody, IgG, competing with IgE was reported as one of the potential mechanisms that have been identified through immunotherapy [20–24]. In our study, the increase in the concentrations of OVA-specific IgG2a in OVA/HSA-sensitized mice treated with OVA–DT was expected because we found out that OVA–DT treatment increased the production of IFN- γ in cultures of lymphocytes from OVA-sensitized mice in the previous study [16].

B cells can not only capture antigen directly via surface Ig's, but also capture antigen indirectly by Ig's bound to their receptors [7, 17, 25, 26].

IgG is generally known to combat infections by neutralizing and opsonizing micro-organisms and their products, rather than being used to capture antigen. Indeed, Fc γ RII of B cells do not get internalized after binding with IgG complexes [27]. However, IgE rapidly becomes bound to neighbouring antigen-presenting cells (APCs) including B cells, and then participate in antigen capture [28, 29]. These facts may support a possibility that OVA–DT will affect B cells expressing OVA-specific IgE more than those expressing OVA-specific IgG1, IgG2a or IgG3.

In our result, there is no positive proof that B cells isolated from OVA/HSA-sensitized mice express OVA-specific IgE. However, the cells that we used were IgE-expressing B cells but not B cells expressing other Ig isotypes, and were killed by OVA–DT in the presence of mouse anti-OVA serum but not mouse anti-HSA serum.

Therefore, we assume that OVA–DT targets only B cells expressing OVA-specific IgE and kills them by IgE-mediated cytotoxicity of OVA–DT.

The cytotoxic effect of OVA–DT on OVA-specific memory B cells was also not investigated in this study. However, it was demonstrated that the decrease in OVA-specific IgE found in OVA/HSA-sensitized mice treated with OVA–DT was also shown in the serum of the mice boosted with the same allergens at day 26. This suggests the possibility that OVA–DT killed OVA-specific memory B cells and thereby induced tolerance of B cells producing OVA-specific IgE.

Lustgarten et al. [13] reported that an immunotoxin composed of anti-IgE and an A chain of ricin eliminates IgE-bearing B cells, and Slater et al. [14] also reported that the immunotoxin kills mast cells. However, because the immunotoxin does not target selectively allergen (or antigen)-specific IgE-bearing B cells, it may damage other cells expressing IgE on their surfaces and thereby influence the host defence associated with IgE antibodies that are induced by some parasites.

OVA–DT can also induce the cytotoxic damage to APCs and other phagocytic cells because the cells capture foreign materials by phagocytose and/or pinocytose or by OVA-specific Ig's [30]. However, IgE-mediated antigen capture of

B cells is extremely efficient because the required antigen concentrations are 1000 times less than capturing antigen through pinocytosis or phagocytosis [31, 32].

Indeed, an exceeding dose of OVA–DT (600 μ g/mouse) appeared to kill the mice (data not shown), but the dose of OVA–DT (100 μ g/mouse) used in this experiment did not kill mice and caused no systemic damages in the immune system of the mice. The evidence that immune systems of OVA/HSA-sensitized mice were not suppressed by the treatment of 100 μ g of OVA–DT is supported by the result that OVA/HSA-sensitized mice treated with OVA–DT died from HSA-induced anaphylaxis and did not die from OVA-induced anaphylaxis.

In conclusion, the therapeutic effect of OVA–DT was specific to OVA-induced allergy in spite of the fact that the mice were sensitized with a mixture of OVA and HSA. In addition, the suppression seemed to be valid even until the mice were boosted.

Reasons for the suppression thus could be assumed from the results that OVA–DT increases the production of OVA-specific IgG2a and IgG3, and decreases the production of OVA-specific IgE by killing B cells bearing OVA-specific IgE on their surfaces.

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