Lack of antigen-specific immune responses in anti-IL-7 receptor α chain antibody-treated Peyer's patch-null mice following intestinal immunization with microencapsulated antigen

Jun Kunisawa[1,2], Ichiro Takahashi[1,3], Akiko Okudaira[1,2], Takachika Hiroi[1], Kazufumi Katayama[1,2], Teruko Ariyama[1,2], Yasuo Tsutsumi[2], Shinsaku Nakagawa[2], Hiroshi Kiyono[1] and Tadanori Mayumi[2]

[1] Department of Mucosal Immunology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan
[2] Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[3] Department of Preventive Dentistry and Host Defense, Hiroshima University, Graduate School of Biomedical Sciences, Hiroshima, Japan

Peyer's patches (PP) represent a well-characterized inductive site in gut-associated lymphoid tissue that actively acquires antigens from the intestinal lumen. It was reported that organized PP are not required for antigen-specific IgA responses induced by oral immunization with soluble antigen mixed with the mucosal adjuvant, cholera toxin. However, the role of PP in the induction of mucosal and systemic immune responses remains to be clarified in the case of particulate antigen. Here, we created PP-null mice by treating them with monoclonal anti-IL-7 receptor α chain (IL-7Rα) antibody during gestation and then immunized with antigen-encapsulated poly-lactic acid (PLA) microspheres. Brisk OVA-specific antibody responses were noted in serum and fecal extracts of normal mice following direct intestinal immunization with OVA in PBS (OVA-PBS) as well as in PLA-microspheres (OVA-MS). Antibody production was similarly elevated in PP-null mice immunized with OVA-PBS via direct injection into the intestinal tract. In contrast, OVA-specific antibody responses were dramatically decreased in both serum and fecal extracts collected from PP-null mice immunized intestinally with OVA-MS. These results were further supported by the number of OVA-specific antibody-forming cells detected in the spleen and intestinal lamina propria. PP deficiency also resulted in the reduction in OVA-specific Th1/Th2 cell responses in the spleen and mesenteric lymph nodes of mice intestinally immunized with OVA-MS. These results suggested that organized PP do, in fact, play a crucial role in the induction of antigen-specific immune responses against ingested particulate antigen.

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1 Introduction

The intestinal immune system protects the host from being invaded and damaged by toxins, pathogenic microorganisms and allergens [1, 2]. In particular, Peyer's patches (PP) are the primary inductive sites in gut-associated lymphoid tissue that actively acquire antigens from the intestinal lumen via the specialized epithelial cells, M cells, residing in the dome epithelium of PP [3–5]. Powerful bacterial-derived adjuvants (*e.g.* cholera toxin and heat-labile toxin) or novel delivery systems (*e.g.* liposome and microsphere) were frequently tested for the induction of antigen-specific immune responses [6–9]. These previous studies have attempted to effectively activate Th1 and Th2 cells, IgA committed B cells and antigen-presenting cells (APC) in PP to induce mucosal and systemic immune responses.

Recently, however, it was reported that organized PP might not be required for the activation of the IgA response in the gastrointestinal tract since antigen-specific mucosal IgA antibody responses were induced

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Abbreviations: PP: Peyer's patch **IL-7Rα:** IL-7 receptor α chain **PLA:** Poly-lactic acid **OVA-MS:** PLA-microsphere containing OVA **OVA-PBS:** OVA in PBS **PBS-T:** PBS containing 0.05% Tween 20 **SP:** Spleen **MLN:** Mesenteric lymph nodes **iLP:** Intestinal lamina propria **AFC:** Antibody-forming cells

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without organized PP by oral immunization with soluble protein antigens mixed with mucosal adjuvant, cholera toxin [10]. It was suggested that mesenteric lymph nodes (MLN) might behave as the substitute inductive site in these lymph toxin β receptor-Ig-treated PP-null mice. However, the role of PP on the induction of mucosal and systemic immune responses remains to be clarified in the case of orally administered particulate antigens including antigen-encapsulated microspheres and gene-modified bacteria-based vaccines that appear to be effectively taken up by PP via M cells [11–14]. In particular, the poly-lactic acid (PLA) microsphere is a well-characterized microsphere and considered to be a good candidate for a mucosal vaccine vehicle due to its ability and biodegradable characteristics [15–17].

The primary aim of this study was to investigate whether antigen sampling and processing by PP are essential for the induction of antigen-specific mucosal and systemic immune responses following intestinal immunization with antigen-encapsulated PLA-microsphere.

2 Results

2.1 Characterization of PLA-microsphere containing OVA

In the first study, we examined the physiological characteristics of PLA-microsphere containing OVA (OVA-MS) using in this study. Scanning electron microscopy (SEM) analysis revealed that OVA-MS used in this study was spherical and the diameter was about 8 μm (Fig. 1A). The OVA release test showed that a burst release at early stage and stable release at late stage (Fig. 1B).

2.2 Characterization of anti-IL-7R antibody-treated PP-null mice

In the next study, we characterized PP-null mice used in this study. Pregnant mice were injected with anti-IL-7Rα monoclonal antibody to obtain PP-null mice [18, 19]. As shown in Fig. 2, the offspring mice were deficient in the organized PP, whereas normal mice showed eight to ten PP in the small intestine (Fig. 2A, B). In contrast to PP-null mice, normal mice exhibited isolated lymphoid follicles that were composed of a large B220$^+$ area as recently reported (Fig. 2C, D) [20]. In addition, the PP-null mice possessed comparable numbers and populations of immunocompetent cells in bone marrow, thymus, spleen, inguinal lymph node and MLN [18], and had normal serum Ig concentration and isotype profiles (Table 1).

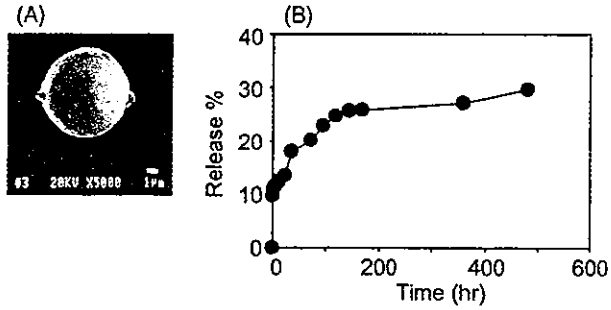

Fig. 1. Characterization of OVA-MS. Scanning electron microphotograph (×5,000) were obtained by standard method (A). Release profiles of OVA from PLA-MS were examined as described in Sect. 4 (B).

2.3 Induction of antigen-specific serum IgG and fecal IgA responses

We next examined and compared OVA-specific antibody responses in serum and fecal extracts of normal and PP-null mice. Mice were immunized with antigen in soluble form or in MS by direct administration into duodenum (intestinal immunization). As shown in Fig. 3A, high levels of antigen-specific antibody production was effectively induced by intestinal immunization compared with oral immunization, and the addition of a mucosal adjuvant such as cholera toxin was not required for the induction of antigen-specific antibody production in this immunization regimen (Fig. 3A) [21, 22]. Furthermore, intestinal immunization can also avoid antigen degradation and denaturation in stomach, so that we could directly compare any possible contribution of PP to the induction of antigen-specific immune responses to two distinct, *i.e.* OVA in PBS (OVA-PBS) and OVA-MS, antigen preparations without the other undesirable effects. When normal mice were immunized by direct intestinal administration with either OVA-PBS or OVA-MS, brisk OVA-specific

Table 1. Serum Ig isotype in PP-null and normal mice[a]

Isotypes	PP-null mice	Normal mice
IgG	2.249 ± 0.353	2.469 ± 0.283
IgA	0.148 ± 0.015	0.185 ± 0.019
IgG1	0.781 ± 0.237	0.701 ± 0.062
IgG2a	0.291 ± 0.011	0.299 ± 0.012
IgG2b	0.371 ± 0.049	0.523 ± 0.060
IgG3	0.115 ± 0.012	0.138 ± 0.016

a) Data represent mean ± SE of concentration (mg/ml) from three separate experiments (four mice per group).

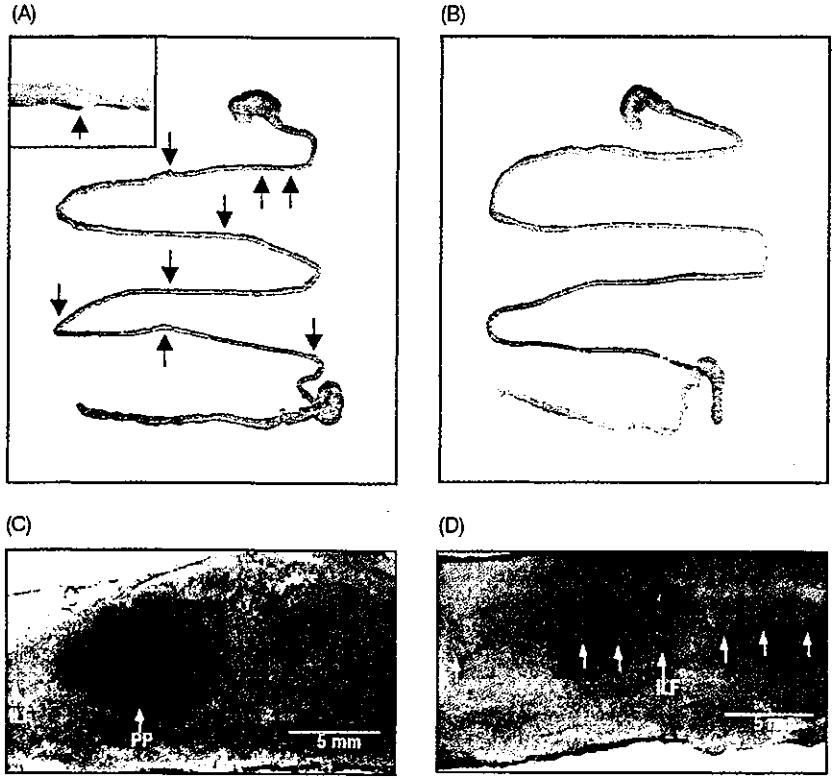

Fig. 2. Microscopic analysis of lymphoid organs in the small intestine of PP-null and normal mice. Intestines isolated from normal (A) and PP-null (B) mice are shown. Arrows indicate the position of organized PP (A). Localization of isolated lymphoid follicles (ILF) and PP in the small intestine of normal (C) and PP-null (D) mice are indicated by arrows.

serum IgG and fecal IgA responses were induced (Fig. 3B, C). Furthermore, it was interesting to note that similar levels of serum IgG and fecal IgA responses were also induced in PP-null mice intestinally immunized with OVA-PBS. In contrast, OVA-specific antibody responses were rarely detected in either the serum or fecal extracts from PP-null mice intestinally immunized with OVA-MS (Fig. 3B, C). From the results of the analysis of antigen-specific antibody production in mice intraperitoneally immunized with two distinct forms of OVA, it could be that antigen did not leak from the intestine after the intestinal immunization. Thus, intraperitoneal immunization with 100 μg OVA in either PBS or MS resulted in the induction of OVA-specific serum IgG (Fig. 3D) but not fecal IgA responses (data not shown). This point was further supported by the results of intraperitoneal immunization with reduced amounts of OVA-PBS (1, or 10 μg), which did not induce any OVA-specific serum IgG responses, whereas intraperitoneal immunization with MS containing 10 μg OVA, but not 1 μg, induced OVA-specific serum IgG responses (Fig. 3D). These findings also indicate that organized PP deficiency resulted in reduced antigen-specific antibody production in both serum and fecal extract following intestinal immunization with antigen in MS.

These findings were further confirmed by ELISPOT assay of OVA-specific antibody-forming cells (AFC). Following direct intestinal immunization of OVA-PBS or OVA-MS, normal mice possessed a large number of OVA-specific AFC in the spleen (SP) and intestinal lamina propria (iLP) (Fig. 4). On the other hand, organized PP-deficient mice had a reduced number of OVA-specific AFC in both the SP and iLP when the mice were intestinally immunized with OVA-MS, but not OVA-PBS (Fig. 4). Thus, the number of OVA-specific AFC noted in PP-null mice immunized with OVA-PBS was similar to that seen in normal mice.

We also assessed OVA-specific serum IgG subclass responses. Significant IgG1 responses were observed in normal and PP-null mice following intestinal immunization with OVA-PBS (Fig. 5A). In addition, intestinal immunization of normal mice with OVA-MS induced antigen-specific IgG2a and IgG2b responses as well as dominant IgG1 isotype responses (Fig. 5B). Corresponding to the

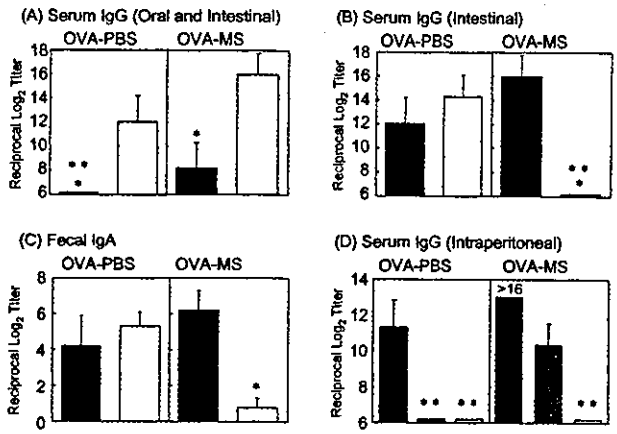

Fig. 3. OVA-specific antibody production in serum and fecal extract induced by immunization with OVA-PBS or OVA-MS. Normal mice were orally (solid column) or intestinally (open column) immunized with OVA-PBS (left) or OVA-MS (right) at days 0, 21, and 42. At 7 days after the final immunization, OVA-specific antibody production in serum was measured (A). Normal mice (solid column) or PP-null mice (open column) were intestinally immunized with OVA-PBS (left) or OVA-MS (right) at days 0, 21 and 42. At 7 days after the final immunization, serum (B) and fecal extract (C) were collected for the ELISA analysis of anti-OVA specific IgG and IgA productions, respectively. Normal mice were intraperitoneally immunized with titrated amounts of OVA-PBS (left) or OVA-MS (right) at days 0, 21, and 42. At 7 days after the final immunization of the normal mice with 100 μg (black column), 10 μg (gray column), or 1 μg (open column) of OVA in PBS (left) or in MS (right), OVA-specific IgG production in serum was determined by ELISA (D). The error bars indicate the mean ± SE for four mice analyzed separately in triplicate assays. *$p<0.05$ [compared with oral immunization (A) or with normal mice (B and C)], **reciprocal log2 titer <6.

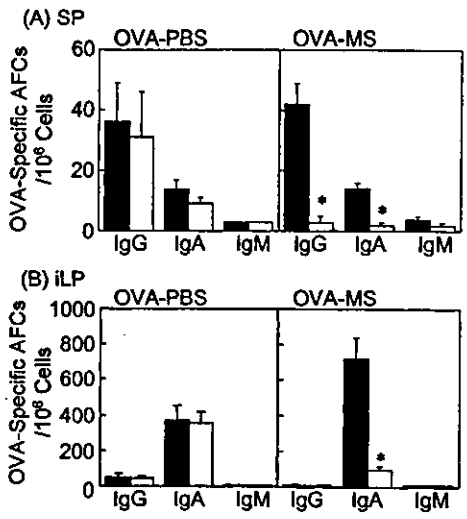

Fig. 4. OVA-specific AFC in normal (solid column) and PP-null mice (open column) following intestinal immunization of OVA-PBS and OVA-MS. Mice were intestinally immunized with OVA-PBS or OVA-MS at days 0, 21 and 42. At 7 days after the final immunization, mononuclear cells were isolated from the SP (A) and iLP (B) of normal (solid column) and PP-null (open column) mice, and then evaluated for OVA-specific IgG, IgM and IgA AFC by ELISPOT assay. Values presented are representative of three separate experiments. *$p<0.01$ (compared with normal mice).

lack of OVA-specific IgG production (Fig. 3 and 4), all IgG subclasses of OVA-specific antibody production were deficient in PP-null mice intestinally immunized with OVA-MS (Fig. 5B).

2.4 Antigen-specific CD4⁺ T cell responses

Given that organized PP appeared to be crucial site for the induction of OVA-specific mucosal and systemic antibody responses following intestinal immunization with OVA-MS, we felt that it was important to analyze antigen-specific CD4⁺ T cell responses that provide helper signal for the generation of OVA-specific antibodies. We initially examined OVA-induced proliferative responses of CD4⁺ T cells in the MLN and SP. High levels of OVA-specific CD4⁺ T cell proliferative responses were noted in the MLN of normal mice intestinally immunized with OVA-PBS or OVA-MS (Table 2). However, these proliferative responses of antigen-specific CD4⁺ Th cells were significantly decreased in the MLN of PP-null mice intestinally immunized with OVA-MS (Table 2). Similar tendencies were also noted in the splenic CD4⁺ T cells (Table 2). These data suggest that intestinal immunization with the soluble-form of OVA can induce and activate antigen-specific CD4⁺ T cells via both the PP-independent and PP-dependent pathways. In contrast,

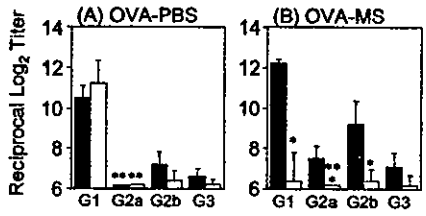

Fig. 5. Serum IgG subclass responses to OVA in mice intestinally immunized with OVA-PBS or OVA-MS. Normal mice (solid column) or PP-null mice (open column) were intestinally immunized with OVA-PBS (A) and OVA-MS (B) as described in Fig. 1. Serum samples were collected at day 49 and examined for OVA-specific serum IgG subclass responses by ELISA. Error bars indicate the mean ± SE for four mice analyzed separately in triplicate assays. *$p<0.05$ (compared with normal mice); **reciprocal log2 titer <6.

Table 2. Comparison of proliferative responses of OVA-specific CD4+ T cells isolated from intestinally immunized PP-null and normal mice with OVA-MS or OVA-PBS[a)]

	PP-null mice	Normal mice
MLN		
OVA-PBS	9333 ± 844	10779 ± 1323
OVA-MS	3568 ± 210*	11495 ± 406
SP		
OVA-PBS	10268 ± 765	9880 ± 765
OVA-MS	2920 ± 121*	18055 ± 1264

a) CD4+ T cells were purified from the MLN and SP of normal and PP-null mice and cultured with OVA (1 mg/ml) in the presence of irradiated syngenic spleen cells for 96 h. During the last 8 h of incubation, 1.0 μCi [^{3}H]thymidine was added. Results are expressed as the means ± SE (cpm) from four mice per group and from three experiments. *$p<0.01$ (compared with normal mice).

antigen-specific CD4+ T cells were mainly induced and activated via the PP-dependent pathway following intestinal immunization of the antigen-encapsulated microspheres.

In the next experiment, we evaluated the quality of Th responses induced by OVA-PBS or OVA-MS. CD4+ T cells isolated from the MLN of mice intestinally immunized with OVA-PBS were found to secrete a large amount of Th2-type (IL-4) cytokine, but not Th1-type (IFN-γ) cytokine (Fig. 6A, B). In contrast, intestinal immunization of normal mice with OVA-MS induced high level of a Th1-type (IFN-γ) cytokine production in addition to a Th2-type (IL-4) cytokine from antigen-specific CD4+ T cells (Fig. 6A, B). These tendencies were also noted when splenic CD4+ T cells were examined (Fig. 6C, D). In the case of PP-null mice, these Th1 and Th2 cytokine productions were rarely observed following direct intestinal immunization of OVA-MS (Fig. 6A).

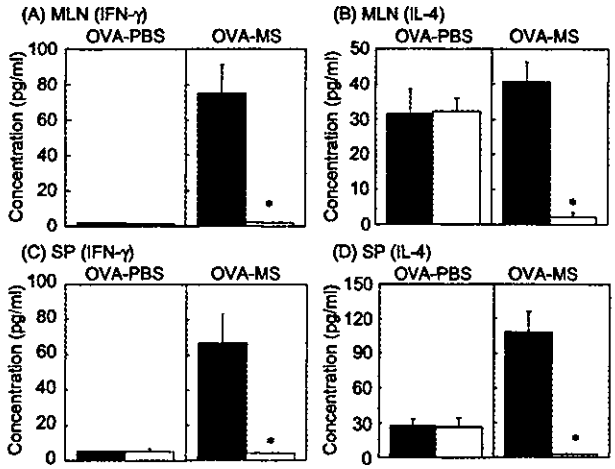

Fig. 6. Analysis of Th1 (IFN-γ) and Th2 (IL-4) cytokine productions by CD4+ T cells in the MLN and SP of intestinally immunized normal (solid column) and PP-null (open column) mice with OVA-PBS or OVA-MS. CD4+ T cells from the MLN (A, B) and SP (C, D) of intestinally immunized mice were stimulated *in vitro* with OVA as described in Fig. 4. Culture supernatants were harvested for the analysis of secreted Th1 and Th2 cytokines using the respective cytokine-specific ELISA. Results are expressed as the means ± SE from four mice per group and from a total of three experiments. *$p<0.01$ (compared with normal mice).

3 Discussion

In the present study, we examined the essential role of PP in the induction of antigen-specific mucosal and systemic immune responses by mucosal delivery of particulate-form antigens (*e.g.* microsphere). Our results showed that PP-null mice created by *in vivo* treatment during gestation with monoclonal anti-IL-7Rα antibody failed to elicit antigen-specific CD4+ Th cells and antibody immune responses following intestinal immunization with OVA-MS. M cells, known to exist in the follicle-associated epithelium of PP, are specialized for the sampling and uptake of particulate antigens (including microorganisms, virus and biodegradable microspheres) and subsequent translocation to underlying APC [3, 4, 5, 11–14]. In this respect, microspheres with an 8–10 μm diameter can be effectively taken up by PP [15, 17]. Accordingly, we used an identical size of microsphere containing OVA (OVA-MS) in the present study (Fig. 1A). Although a recent report suggested that M cells also exist on the isolated lymphoid follicles of anti-IL-7Rα-treated PP-null mice [20], our present findings suggests that PP are still important for the induction of antigen-specific CD4+ Th and B cell responses following direct immunization of particulate-form antigen.

In contrast, when intestinally immunized with OVA-PBS, antigen-specific immune responses were elevated in both the mucosal and systemic compartments of both PP-null and normal mice. In a separate study [10], it was reported that immunocompetent T and B cells in MLN compensate for the lack of PP and possess the ability to induce antigen-specific immune responses at distant effector sites, such as the iLP, of lymph toxin β receptor-Ig-treated PP-null mice following oral immunization with soluble protein antigen and the mucosal adjuvant, cholera toxin. Additionally, other PP-null mice have been generated by an adaptation of a different gene-mutation

system [23–25]. Although it was possible to use PP-null mice generated by the gene mutation, these gene-mutation PP-null mice do not possess normal lymphocyte function or normal organogenesis of other secondary lymphoid tissues. In contrast, the *in utero* anti-IL-7Rα-monoclonal antibody-treated PP-null mice used in this study showed the normal lymphocyte numbers and functions in the other tissues [18]. In addition, this study demonstrated that comparable levels and isotype profiles of serum Ig were seen in PP-null mice when compared with wild-type mice (Table 1) and mock monoclonal antibody-treated control mice (data not shown). Thus, it should be emphasized that this PP-null mice system, generated by *in utero* treatment with anti-IL-7Rα monoclonal antibody, is considered to be the best system for analyzing the role of organized PP function in the induction of antigen-specific immune responses against particulate and soluble antigens administered via the gastrointestinal mucosa.

Additionally, there is now increasing evidence that villus epithelium is not an impermeable barrier to macromolecules, but in fact participates in the immune response to orally absorbed antigens [26, 27]. The villus epithelium allows translocation of proteins in a native or partially degraded form, so that they can be recovered in the blood [28]. Further, intestinal epithelial cells may serve as APC since they have been shown to express MHC class II [29] and co-stimulatory molecules, including CD86 and ICAM-1 [30]. Thus, it is possible that ingested soluble antigen might be taken up via villus epithelial cells for presentation to the underlying mucosal Th1 and Th2 cells, subsequent to the induction of antigen-specific immune responses. In addition, dendritic cells (DC) in the iLP were recently reported to transport the remnants of apoptotic intestinal epithelial cells to the T cell area of the MLN [31]. Thus, a soluble form of antigen can be presented to the mucosal network to induce the antigen-specific immune response without PP involvement. Furthermore, recent studies have indicated that $CD18^+$ phagocytes expressing appropriate tight junction molecules are capable of directly sampling bacteria from gut lumen [32, 33]. These $CD18^+$ phagocytes, including mucosal DC, may participate in the initiation for the induction of antigen-specific immune responses in PP-null condition.

Our present study also showed that both intestinally administered OVA-MS and OVA-PBS induced antigen-specific proliferative responses in $CD4^+$ T cells isolated from the MLN and SP of normal mice (Table 1). Among these OVA-specific $CD4^+$ T cell responses, the predominant Th2 responses were elevated by immunization with OVA-PBS. In contrast, both Th1 and Th2 responses were generated in normal mice intestinally immunized with OVA-MS (Fig. 6). Interestingly, distinct populations of DC were found in the subepithelial dome and T cell regions of the PP [34–38]. $CD8^+$ lymphoid DC expressing CCR7 were found in the T cell-rich interfollicular region, which preferentially induces Th1 responses. In contrast to these lymphoid DC, $CD11b^+$ myeloid DC in the subepithelial dome region expressed both CCR6 and CCR7 and induced Th2 responses [35, 37]. Moreover, other studies have demonstrated that DC in the intestinal wall acquire soluble protein antigen and then migrate to peripheral lymph, carrying the antigen in a form that can be presented to sensitize $CD4^+$ Th cells in an MHC class II-restricted manner [31, 39, 40]. These distinct DC in mucosal tissue may influence on the profile of Th1 and Th2 $CD4^+$ T cell responses induced by intestinal immunization with OVA-MS or OVA-PBS.

In summary, our data suggest that PP are necessary for the induction of particulate antigen-induced Th1/Th2 $CD4^+$ T cells as well as IgA and IgG antibody responses. Furthermore, the nature of antigen (*e.g.* soluble and particulate forms) will determine the entry and processing sites of antigen (*e.g.* PP-dependent and independent pathways) in the intestine, which will in turn influence the outcome of subsequent antigen-specific immune responses.

4 Materials and methods

4.1 Mice

Female BALB/c mice (7–10 weeks old) and pregnant BALB/c mice were purchased from Japan SLC (Shizuoka, Japan).

4.2 Development of PP-null mice using anti-IL-7Rα antibody

Anti-IL-7Rα antibody-producing hybridoma, A7R34, was kindly provided by Dr. Shin-ichi Nishikawa (Kyoto University, Japan) [18, 19]. Pregnant BALB/c mice were injected intravenously and subcutaneously with 1 mg anti-IL-7Rα antibody, respectively, at 14.5 days post coitus as previous described [18]. We confirmed the disruption of organized PP and existence of isolated lymphoid follicles in offspring using immunohistochemical analysis as described previously [20] (Fig. 2A).

4.3 Preparation and characterization of OVA-MS

PLA microspheres were prepared by the w/o/w emulsion solvent evaporation method [41]. Briefly, OVA (Sigma, St. Louis, MO) solution (100 mg/ml in PBS) was emulsified with 200 mg/ml PLA (Wako, Osaka, Japan) in methylene chloride for 5 min using a microhomogenizer (Microtec Niti-on,

Chiba, Japan). The w/o emulsion was then added to 0.25% polyvinyl alcohol solution (Wako) and homogenized to produce a stable w/o/w double emulsion. The dispersion was gently agitated by stirring for 4 h at room temperature. The microspheres were washed and collected by centrifugation at 10,000 rpm for 20 min. The diameter and morphology of obtained OVA-MS were examined by scanning electron microscopy (SEM).

To characterize the release profile of OVA from PLA-MS, OVA-MS were suspended in PBS (100 mg/ml) and stirred as described previously [15]. At predetermined intervals, the suspension was taken as a sample, centrifuged (12,000 rpm, 10 min) and the concentration of OVA in the supernatant was determined by HPLC.

4.4 Immunization

Age-matched (7–10 weeks old) normal mice and PP-null mice were fasted overnight before undergoing a celiotomy under light anesthesia. To introduce the antigen, 200 μl solution containing 100 μg OVA in PBS (OVA-PBS) or encapsulated in PLA microspheres (OVA-MS) was then injected into the duodenal lumen of each mouse before the abdominal muscle wall and skin were closed using a nylon suture [21, 22]. This direct intestinal immunization was carried out on days 0, 21 and 42. As controls, mice were orally or intraperitoneally immunized with same OVA preparations at same schedule.

4.5 Isolation of mononuclear cells

Single cells were obtained from the SP, MLN and iLP as previously described [10, 42–44]. Briefly, mononuclear cells from the SP and MLN were isolated by the mechanical dissociation method using gentle teasing through a stainless steel screen. Following the removal of PP, iLP mononuclear cells were isolated by the enzymatic dissociation procedure using collagenase type IV (Sigma). The lymphocytes then were obtained at the interface between the 40% and 75% layers of a discontinuous Percoll gradient (Amersham Pharmacia Biotech, Piscataway, NJ).

4.6 Detection of total serum Ig level and antigen-specific antibody responses in serum and fecal extract

For detection of total serum Ig levels in sera, sandwich ELISA was employed as described previously [44]. To examine OVA-specific antibody production, standard OVA-specific ELISA and ELISPOT assay were performed as previously described [10, 42–44].

4.7 Proliferative responses of antigen-specific CD4$^+$ T cells

Seven days after the final immunization, lymphocytes were obtained from the SP and MLN. CD4$^+$ T cells were then purified using anti-mouse CD4 (L3T4)-coupled microbeads and a magnetic-activated cell sorter column (Miltenyi Biotec, Sunnyvale, CA) [42, 43]. Purified CD4$^+$ T cells were cultured with 1 mg/ml OVA in the presence of irradiated (3,000 rad) splenic feeder cells at 37°C for 96 h [6, 10, 42, 43]. To measure cell proliferation, 1 μCi [^{3}H]thymidine was added to individual culture wells 8 h before termination, and the uptake of [^{3}H]thymidine by dividing cells was determined by scintillation counting.

4.8 Cytokine analysis by ELISA

Cytokine levels in culture supernatants of antigen-stimulated CD4$^+$ T cells were determined by a cytokine-specific ELISA [6, 10, 42–44]. Briefly, CD4$^+$ T cells obtained from the SP and MLN of the immunized mice were cultured at a density of 1×10^6 cells/ml with 1 mg/ml of OVA in the presence of irradiated (3,000 rad) splenic feeder cells. Culture supernatants were harvested 96 h after incubation, and the levels of Th1 (IFN-γ) and Th2 (IL-4) cytokines were determined by cytokine-specific ELISA (Amersham Life Sciences, Arlington Heights, IL). The concentration of cytokines was calculated from the standard curves obtained according to the manufacturer's instructions.

4.9 Statistics

The results were compared using Student's *t*-test and statistical significance was established at the $p<0.05$.

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Correspondence: Tadanori Mayumi, Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
Fax: +81-6-6879-8179
e-mail: mayumi@phs.osaka-u.ac.jp

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Immunosuppressive properties of human amniotic membrane for mixed lymphocyte reaction

M. UETA*†, M.-N. KWEON†, Y. SANO*, C. SOTOZONO*, J. YAMADA*, N. KOIZUMI*, H. KIYONO† & S. KINOSHITA* *Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto and †Department of Mucosal Immunology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

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SUMMARY

The combination of allograft limbal transplantation (ALT) and amniotic membrane transplantation (AMT) has been applied in the treatment of severe ocular surface diseases. The beneficial effect of this combination has been thought to result from possible immunosuppressive ability of amniotic membrane (AM). However, the mechanisms of any such ability remain unknown. In this study, we investigated whether human AM has the ability to suppress allo-reactive T cell responses *in vitro*. For mixed lymphocyte reaction (MLR), lymphocytes isolated from lymph nodes of C57BL/6 mice (Mls1^b, V$_\beta$6$^+$) were cultured with irradiated splenocytes from DBA/2 mice (Mls1^a, V$_\beta$6$^-$) with or without human AM. For carboxyfluorescein diacetate succinimidyl ester (CFSE) experiments, responder lymph node cells were labelled with a stable intracellular fluorescent dye and cultured with irradiated stimulator cells. The ratio of responder V$_\beta$6$^+$ T cells was then determined by FACS analysis, and the division profiles of responder V$_\beta$6$^+$ T cells were analysed by CFSE content. Furthermore, Th1 and Th2 cytokine synthesis by allo-reactive T cells in MLR culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA). Addition of AM to the MLR culture resulted in the significant inhibition of thymidine incorporation compared with control culture lacking AM. The population of responder CD4$^+$V$_\beta$6$^+$ T cells was significantly reduced in the AM-treated culture in comparison to control. CFSE analysis revealed less division and lower proliferation of responder CD4$^+$V$_\beta$6$^+$ T cells in cultures with AM than without. In addition, allo-rective T cell synthesis of both Th1 (IL-2 and IFNγ) and Th2 (IL-6 and IL-10) type cytokine was significantly decreased in the presence of AM. These results indicate that human AM has the ability to suppress allo-reactive T cells *in vitro*. This inhibitory effect likely contributes to the success of the ALT-AMT combination.

Keywords amniotic membrane (AM) carboxyfluorescein diacetate succinimidyl ester (CFSE) mixed lymphocyte reaction (MLR) cytokine flow cytometry

INTRODUCTION

When pathological insults destroy limbal epithelial stem cells, the corneal surface invariably heals with conjunctival epithelial ingrowth (conjunctivalization), neovascularization, chronic inflammation, and recurrent or persistent corneal epithelial defects [1–5]. These pathological conditions constitute the newly established disease called limbal (stem cell) deficiency. This can result from several causes, including total destruction of the limbal stem cell population by chemical or thermal injury, Stevens–Johnson syndrome, multiple surgical or cryotherapy procedures at the limbal region, contact lens wear and severe microbial infection [6,7]. Most cases of limbal deficiency require the use of limbal auto-grafts or allografts for corneal surface reconstruction [8–11]. Therefore, allograft rejection of transplanted limbal tissues is conventionally combated by administering oral steroid and cyclosporin [9,10]. One newly emerging approach in the treatment of limbal deficiency is the use of amniotic membrane transplantation (AMT), first introduced by Kim and Tseng [12]. Amniotic membrane (AM), a thin semitransparent tissue forming the innermost layer of the fetal membrane [13], has a thick continuous basement membrane with a full complement of collagen types IV and V and laminin, the main basement membrane components [14]. AM has been used clinically to promote epithelialization in burns and skin ulcers, or as dressing in wounds or skin grafts [15–17]. It also has been proven suitable for epithelial cell culturing [18,19].

Correspondence: Mayumi Ueta, Department of Ophthalmology, Kyoto Prefectural University of Medicine, Hirokoji, Kawaramachi, Kamigyoku, Kyoto 602–0841, Japan.
E-mail: mueta@ophth.kpu-m.ac.jp

Previously, treatments such as simple penetrating keratoplasty or allograft limbal transplantation could not produce satisfactory results in Stevens–Johnson syndrome, pseudopemphigoid and chemical burns. However several groups have recently reported that the combination of allograft limbal transplantation (ALT) and AMT was useful for reconstructing the ocular surface in these diseases [20–22]. Accompanying conditions, including severe dry eye, lack of corneal stem cells, trichiasis, and persistent ocular surface inflammation, exacerbate these diseases' refractoriness to the treatment. However, it has been hypothesized that AMT effectively facilitates epithelialization and reduces inflammation and scarring, desirable effects for promoting the success of ALT. Transplanted AM also seems to promote normal conjunctival epithelialization, in addition to preventing excessive subconjunctival fibrosis formation. Type IV collagen has been recognized histochemically in conjunctival, but not in corneal epithelial, basement membrane [12]. The collagen in AM therefore probably serves as a suitable substrate for conjunctival epithelialization. In fact, AMT has been regarded as substrate transplantation.

The beneficial effect of the ALT-AMT combination is also thought to result from possible immunosuppressive effect of AM, since placental tissues, including AM, have been shown to suppress the semiallo immune response against the fetus [23,24]. However, the immunological effects of AMT are not yet fully understood. In this study, we investigated whether human AM has the ability to suppress T cell proliferation *in vitro*. Our findings demonstrated that human AM inhibited allo-reactive T cell responses, including proliferation, cell division and Th1 and Th2 cytokine synthesis. Further, novel results obtained by the study suggest the interesting possibility that soluble inhibitory factor secreted by human AM has the ability to suppress allo-reactive T cells *in vitro*. It is likely that the immunosuppressive function of human AM contributes to the success of the ALT-AMT combination.

METHODS

Mice

C57BL/6 (H-2^b, Mls1^b, $V_\beta 6TCR^+$) and DBA/2 (H-2^d, Mls1^a, $V_\beta 6TCR^-$) mice (Shimizu Laboratory Supplies, Kyoto, Japan) 6–12 weeks of age were used for the experiments. These strains differ at the major histocompatibility complex and at numerous minor histocompatibility loci. All animals were housed in the experimental animal facility at Kyoto Prefectural University of Medicine, and received sterilized food and autoclaved tap water. All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Human amniotic membrane preparation

Human AM was obtained as described previously [25–27]. Briefly, human AM was obtained at the time of cesarean section, with proper informed consent. Under sterile conditions, the AM was washed 3 times in 200 ml phosphate-buffered saline (PBS) containing antibiotics (5 ml of 0·3% ofloxacin), washed once in 50% glycerol/DMEM and stored at –80°C in 50% glycerol/DMEM. AM for explant was then thawed, excised at 10 mm × 10 mm and examined *in vitro* for immunosuppressive properties. For some experiments, human AM was pretreated with 100% ethanol. This prefixed human AM was extensively washed with PBS prior to use for *in vitro* experiment.

Cell preparations

Mice were sacrified by cervical dislocation. Lymph node and spleen were then aseptically removed. Lymph node cells and splenic cells, obtained by dissociation using Nylon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA), were suspended in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) [28]. Culture medium comprised 10 mM HEPES, 0·1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1×10^{-5}M 2-mercaptoethanol, 0·1% bovine serum albumin (Sigma, St. Louis, MO, USA), and ITS$^+$ culture supplement (Becton Dickinson Labware).

Culture condition

Human AM on transwell (Corning Incorporatedm New York, NY, USA) was added to the MLR culture of C57BL/6 (Mls1^b, $V_\beta 6TCR^+$) responder and DBA/2 (Mls1^a, $V_\beta 6TCR^-$) stimulator. Lymph node cells (2×10^6) from C57BL/6 mice (as responders) were mixed with gamma-irradiated (20Gly) spleen cells (2×10^6) from histoincompatible DBA/2 mice (as stimulators); the mixture was then added directly to 24 culture wells containing transwell with AM. The positive control was MLR of C57BL/6 responder and DBA/2 stimulator without AM. The negative control culture consisted of C57BL/6 responder and C57BL/6 stimulator cells only.

Proliferation assay

Cultures were incubated for 96 h, including a final 8 h pulsed with tritiated thymidine (3·3 μCi/well). Cells were then divided into 96-well microplates, 200 μl/well, resulting in 1 μCi tritiated thymidine per well, and harvested by the Micro-Mate 196 Cell Harvester (Perkin Elmer Life Science Inc., Boston, MA, USA). Thymidine incorporation was measured by beta-counter (Matrix 9600, Perkin Elmer Life Science Inc.).

CFSE labelling and flow cytometry

The intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes Europe BV, Leiden, the Netherlands) was used to determine cell division in responder cells, as described previously [29,30]. The intracellular fluorescent dye CFSE was used to label cells before *in vitro* culture. The responder lymph node cells were resuspended in PBS with 0·1% BSA, CFSE was then added to make a final concentration of 5 μM. The suspension was vortexed immediately following CFSE addition, then incubated for 10 min in a 37°C water bath. The labelled cells were washed twice with PBS with 0·1% BSA and resuspended in culture medium. Human AM on transwell was then added to MLR of CFSE-labelled C57BL/6 responder and DBA/2 stimulator. After 4-day culture, cells were double-stained with PE-conjugated anti-$V_\beta 6$ mAb and APC-conjugated anti-CD4 mAb (BD PharMingen, San Diego, CA, USA). In addition, the division profiles of responder $V_\beta 6^+$ T cells were analysed based on the CFSE content. Stained cells were analysed on a FACS Calibur (Becton Dickinson, San Jose, California), data were analysed using Cellquest software (Becton Dickinson).

Determination of Th1 and Th2 cytokine levels in MLR culture supernatants

Th1 and Th2 murine cytokine levels in MLR culture supernatants were determined by cytokine-specific ELISA, as described previously [31,32]. Briefly, Nunc MaxiSorp immuneoplates (Nagel Nunc International, Rochester, NY, USA) were coated with

monoclonal anti-IFNγ (BD PharMingen) and left at 4°C overnight. After blocking, samples and serial 2-fold dilutions of standards were added to duplicate wells and incubated overnight at 4°C. The wells were washed and incubated with biotinylated monoclonal anti-IFNγ. After incubation, peroxidase-labelled antibiotin Ab (Vector Laboratories, Burlingame, CA) was added and developed with TMB (Moss, Pasadena, MD). Other cytokine levels [IL-2, IL-4, IL-6 and IL-10 (BD PharMingen)] were determined by the same methods as IFNγ Standard curves were generated using mouse recombinant IFNγ(rIFNγ), rIL-2, rIL-4, rIL-6 and rIL-10 (Endogen, Woburn, MA). IFNγ was measured at a sensitivity of 150 pg/ml, IL-2 at a sensitivity of 300 pg/ml, IL-4 at a sensitivity of 150 pg/ml, IL-6 at a sensitivity of 400 pg/ml and IL-10 at a sensitivity of 800 pg/ml.

To assess human-specific cytokines, ELISA kits specific for IL-4, TGF-β (Amersham Biosciences KK, Tokyo, Japan) and IL-10 (BioSource International, California) were used. Human IL-4 was measured at a sensitivity of 10 pg/ml, human IL-10 at a sensitivity of 30 pg/ml and human TGF-β at a sensitivity of 125 pg/ml.

Data analysis

Data were expressed as mean ± SE, and were evaluated by student's *t*-test using the Excel program.

RESULTS

Human AM inhibits allo-reactive T cell responses in murine MLR

We initially investigated whether human AM suppressed MLR. Human AM on transwell was added to MLR cultures of C57BL/6 responder and histoincompatible DBA/2 stimulator. The positive control was the MLR culture of C57BL/6 responder and DBA/2 stimulator, without AM. The negative control culture consisted of responder and stimulator cells from C57BL6 mice. As expected, the positive control resulted in a high level of thymidine incorporation (2401 ± 272 cpm, Fig. 1). However, it was interesting to note that human AM in transwell significantly inhibited MLR (691 ± 189 cpm). This finding suggested the interesting possibility that human AM in transwell may produce an inhibitory factor that suppresses allo-reactive T cells in MLR. To examine this possibility we used AM fixed with 100% ethanol. Fixed human AM on transwell was added to MLR of C57BL/6 responder and DBA/2 stimulator, and compared with MLR containing untreated AM. Interestingly, MLR cultured with fixed AM showed thymidine incorporation levels (2416 ± 245 cpm) comparable to MLR without AM. MLR cultured with fixed AM was not significantly different from that without AM. Murine MLR was not suppressed by fixed human AM. These results suggest that cultured human AM might produce immunosuppressive factors, and that murine MLR might be suppressed by soluble factors produced by human AM.

Inhibition of responder CD4$^+$V$_\beta$6$^+$ T cells by human AM

To directly determine whether human AM has the ability to suppress responder T cells *in vitro*, we next investigated AM effect on responder T cell proliferation by flow cytometry, using a combination of CFSE and mAbs specific for CD4 and V$_\beta$6. Since stimulator DBA/2 mice have minor lymphocyte-stimulating (Mls) 1^a superantigen [33], CD4$^+$V$_\beta$6$^+$ T cells of responder C57BL6 mice were isolated as Mls1^a superantigen reactive T cells. Results were

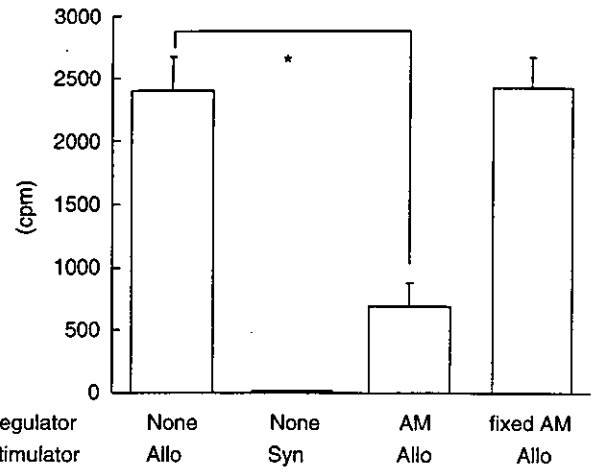

Fig. 1. Inhibition by human AM of proliferative responses of murine allo-reactive mixed lymphocyte reaction (MLR). Human AM was added to the transwell of MLR culture containing C57BL/6 responder and DBA/2 stimulator. Lymph node cells (2×10^6 cells/ml) from C57BL/6 mice (as responders) were mixed with gamma-irradiated (20Gly) spleen cells (2×10^6 cells/ml) from histoincompatible DBA/2 mice (as stimulators). These MLR cultures were then cocultured with human AM in the transwell of 24 wells. These culture plates were incubated for 4 days, during the last 8 h of incubation, ^{3}H-thymidine was added. Positive allogenic control was MLR of C57BL/6 responder and DBA/2 stimulator without human AM. Negative syngenic control consisted of responder and stimulator cells from C57BL/6 without human AM. In some experiments, human AM was prefixed with 100% ethanol, dried, and washed with PBS 4 times. This fixed human AM was added to the transwell of allogenic MLR culture containing C57BL/6 responder and DBA/2 stimulator cells. Radioisotope incorporation detected in 4 separate experiments with triplicate wells is presented as mean cpm ± SEM. (*$P < 0.001$)

expressed as percentages of CD4$^+$V$_\beta$6$^+$ cells among lymphocytes isolated from allogenic MLR with AM, allogenic MLR without AM (positive control), and syngenic MLR without AM (negative control). The mean percentage of CD4$^+$V$_\beta$6$^+$ T cells was 5·5% in allogenic MLR with AM, while the positive and negative control groups contained 12·5% and 2·5% of CD4$^+$V$_\beta$6$^+$ T cells, respectively (Fig. 2a). FACS analysis revealed that the mean percentage of CD4$^+$V$_\beta$6$^+$ T cells in MLR with AM was significantly less than in MLR without AM. The proliferation of responder CD4$^+$V$_\beta$6$^+$ T cells was suppressed by the cultured human AM, possibly via their derived soluble factors.

We next labelled live CD4$^+$V$_\beta$6$^+$ T cells *in vitro* with a stable, fluorescent dye, CFSE that segregates equally between daughter cells upon cell division, enabling fine monitoring of the proliferative history of any T cell present or generated during a response [34]. This system permits simultaneous evaluation of T cell surface markers, and concomitant assessment of cellular activation. T cell division in CFSE-labelled responder cell populations was kinetically analysed. The CFSE histograms were gated for CD4$^+$V$_\beta$6$^+$ T cells on day 4 of the MLR culture, and showed the CFSE fluorescence profile (Fig. 2b). In the positive allogenic MLR culture, the CFSE histograms include large numbers of cells that had undergone more than 3 divisions (left of line). In the syngenic MLR culture without AM, the CFSE histograms revealed the absence of those divided cells. In human AM-treated allogenic MLR cultures, the CFSE histograms demonstrated that smaller numbers of cells had undergone more than 3 divisions,

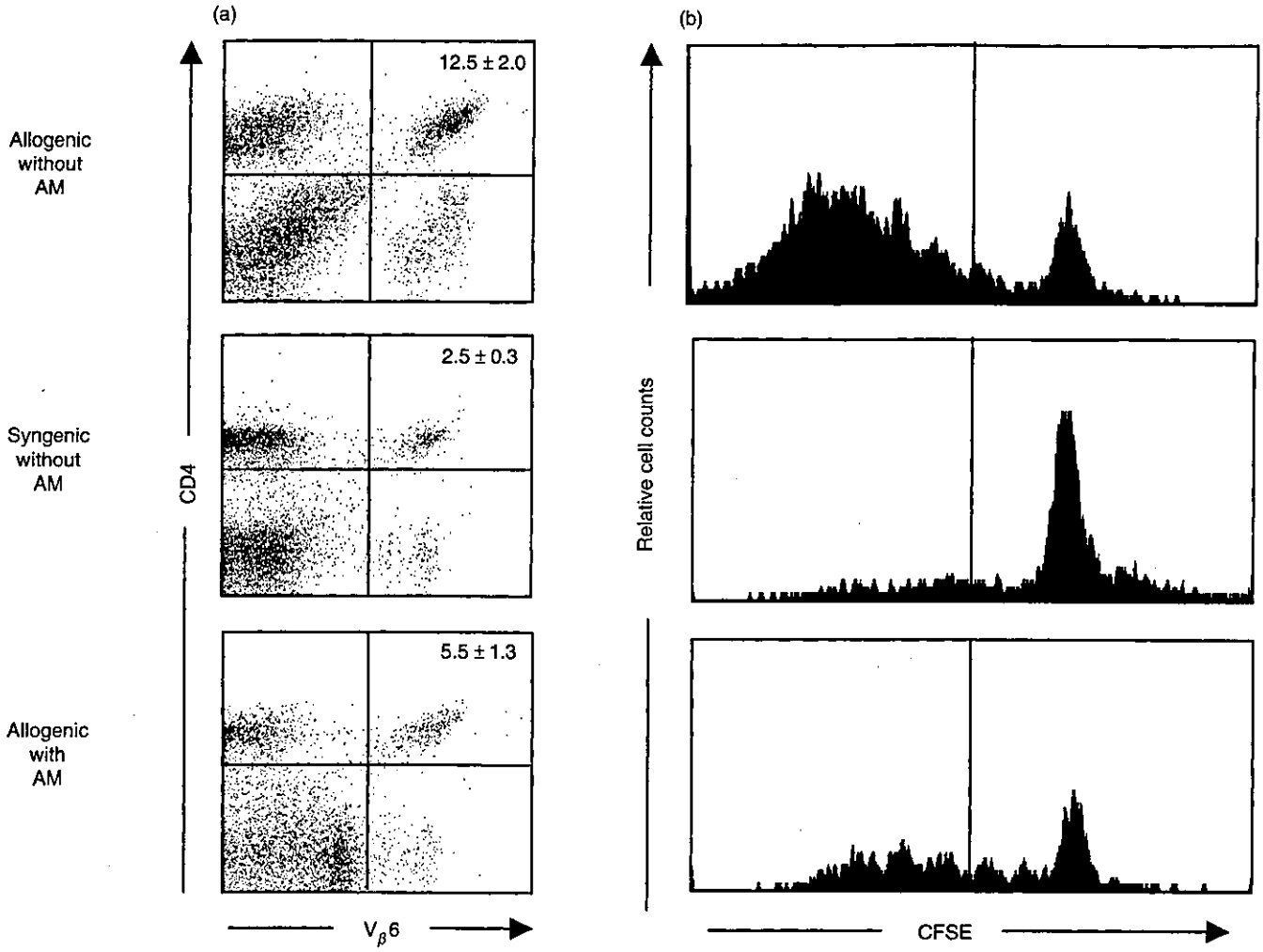

Fig. 2. Suppression of responder $CD4^+$, $V_\beta 6^+$ T cell division by human AM. FACS profile of $CD4^+V_\beta 6^+$ T cells (a) and analysis of responder $CD4^+V_\beta 6^+$ T cell division (b) in murine MLR in the presence or absence of human AM. Lymph node cells (2×10^6) from C57BL/6 mice (as responders) were mixed with gamma-irradiated (20Gly) spleen cells (2×10^6) from histoincompatible DBA/2 mice (as stimulators) in the presence or absence of human AM in the MLR culture transwell. Intracellular fluorescent dye, CFSE, was used to label responder cells before *in vitro* culture. After 4-day culture, cells were double stained with PE-conjugated anti-$V_\beta 6$ mAb and APC-conjugated anti-CD4 mAb. Results of mean percentages from 3 independent experiments are presented as mean (%) ±SEM. Data of CFSE histograms represent of 2 separate experiments.

as compared with the positive control (Fig. 2b). These results demonstrate that the cell division and proliferation of responder $CD4^+V_\beta 6^+$ T cells were suppressed by human AM soluble factors.

Suppression of MLR induced Th1 and Th2 cytokine syntheses by human AM

To examine the effects of AM on MLR-induced T cell cytokine production, the levels of mouse-derived Th1-(IFNγand IL-2) and Th2-(IL-6 and IL-10) type cytokines in MLR culture supernatants were measured on the basis of their maximal production results of time-course study. To this end, IL-2 synthesis was measured in day 2 MLR supernatants, while the other cytokines were measured in day 4 MLR supernatants. Thus, the culture supernatants from the positive MLR control group contained high amounts of both Th1 and Th2 type cytokines. It should be noted that levels of IFNγ, IL-2, IL-6 and IL-10 synthesis decreased significantly when AM was added to the MLR culture transwell (Fig. 3). Moreover, undetectable levels of Th1 (IFNγ, IL-2) or Th2 (IL-6 and IL-10)-type cytokines were produced in the syngenic MLR without human AM group (negative control). Taken together, Th1 (IL-2, IFNγ) and Th2 (IL-6, IL-10)-type cytokines produced by allogenic MLR were significantly inhibited by the presence of human AM in the transwell. To determine whether known regulatory cytokines possessing inhibitory function were produced by human AM in the MLR culture transwell, we examined levels of human inhibitory cytokines (IL-4, IL-10 and TGF-β) in the human AM transwell. None of these human cytokines were detected in the culture supernatant (data not shown).

DISCUSSION

First, this study shows that human AM is capable of inhibiting allo-reactive T cell response in *in vitro* murine MLR. Murine MLR cultured with human AM in transwells showed significantly

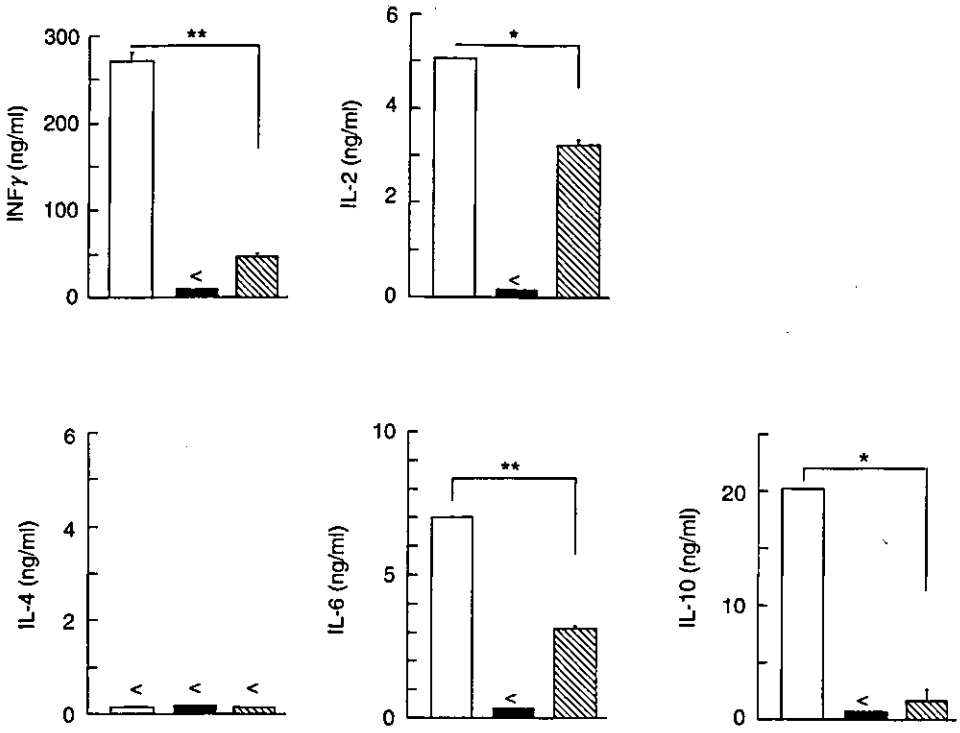

Fig. 3. Inhibition by human AM of Th1 and Th2 cytokine synthesis in murine MLR. Levels of cytokine production in culture supernatants of allogenic MLR with AM (▨), allogenic MLR without AM (□) and syngenic MLR without AM (■) were examined by murine Th1 and Th2 cytokine-specific ELISA. Following 2 day incubation, IL-2 synthesis level was measured. In case of IFNγ, IL-6 and IL-10, supernatants harvested from 4 day cultures were subjected to cytokine-specific ELISA. Data represent mean ± SEM from 1 experiment with duplicated wells. (<not detectable; $*P < 0.01$; $**P < 0.001$)

less thymidine incorporation than that cultured without AM. Next, this study demonstrates that human AM is capable of inhibiting responder $CD4^+V_\beta6^+$ T cells in *in vitro* murine MLR. To this end, FACS analysis revealed that the mean percentage of $CD4^+V_\beta6^+$ T cells in the culture of MLR with AM was significantly less than in the MLR without AM. CFSE analysis also revealed less division of responder $CD4^+V_\beta6^+$ T cells in MLR cultures with AM than without. Furthermore, Th1 and Th2 cytokine production by allo-reactive T cells was also inhibited by human AM in the MLR culture transwell. Taken together, evidence that murine MLR was suppressed by the cultured human AM in transwell indicates that an immunosuppressive factor could be produced by human AM.

Our present results show that cultured human AM tissues inhibit MLR when separated from the mixed lymphocytes by a 0·4-μm pore membrane, indicating that MLR inhibition by human AM is mediated by a soluble factor. To support this view, pretreatment of human AM with 100% ethanol resulted in removal of the inhibitory effect. To our knowledge, this is the first report demonstrating immnosuppressive effects by human AM, possibly via secreted inhibitory factor. Since Streilein *et al.* [35] have proposed that soluble factors secreted by explanted ocular tissues *in vitro* represent the ability of these tissues to create and sustain an immunosuppressive microenvironment *in vivo* within the eye, it is highly possible that our present demonstration of the immunosuppressive effect of human AM *in vitro* allogenic T cell responses reflects in immunoinhibitory function of human AM when transplantated to the eye. It has been demonstrated that murine iris/ciliary body tissues and cells display the ability to suppress murine MLR to which they have been added as regulatory cells [28]. In addition, another previous report showed that rat ciliary body cells were also capable of inhibiting Ag-driven Th lymphocyte proliferation [36]. This inhibitory activity was not species specific, since similar inhibitory effects were observed with bovine and human ciliary epithelial cells [36]. Taking these previous and our present findings together, it is interesting to suggest that human AM may produce a known or unknown soluble factor capable of inhibiting allogenic T cell responses without species specificity.

ALT is rejected easily, sometimes even with immunosuppressive treatment (e.g. steroid). As regards allo-immunogenicity of corneal epithelium, a previous study showed that an intact epithelial structure containing classII MHC-bearing cells (corneal limbus), is capable of inducing proliferation among allo-reactive T lymphocytes [28]. It has been suggested that Langerhans cells migrate out of the limbal tissue to where they can encounter responding lymphocytes for subsequent initiation of MHC-restricted alloantigen reaction [28]. Another previous study showed that full-thickness allogenic corneas induce vigorous delayed hypersensitivity for eventual rejection [37]. Similar results were obtained with allografts of corneal epithelium alone and stromal allografts deprived of endothelium [37]. Furthermore, a previous study has shown that allogenic corneas deprived of epithelium and placed beneath the kidney capsule did not undergo immune rejection during prolonged follow-up [38]. Taken together, these results lead to the reasonable hypothesis that the epithelium is the site primarily responsible for the allo-immunogenicity of heterotopic corneal grafts; and that corneal

epithelium has strong alloimmunogenicity. As well, outside the eye, afferent lymphatic vessels carry antigens in highly immunogenic form to regional lymph nodes in which naive T cell activation first occurs, leading to induction of conventional immunity [39]. The combination of ALT and AMT is more successful for the ocular surgery than ALT only, since AMT is thought to have the beneficial effect of AM's immunosuppressive ability. To this end, our present study directly and experimentally demonstrated that human AM possesses suppressive function that inhibits allo-reactive T cell response using murine MLR system. Further, the result suggested an interesting possibility that human AM may secrete inhibitory factor which can suppress allo-reactive T cell responses.

Previous studies have demonstrated that several factors contribute to the presence of immunological privilege in the eye. Biological fluid obtained from the anterior chamber of the eye has been shown to be immunosuppressive, an effect at least partly explained by the presence of TGF-β [40–43]. It has been demonstrated that mouse and rat iris/ciliary body cells can produce immunosuppressive factors, including TGF-β for the inhibition of MLR [28,36]. Human AM has been shown to express mRNAs for, and produce, TGF-β1 and -β2 [26]. In addition, a previous report has shown that *in vitro*, cytotrophoblasts, a placental tissue, produce IL-10, a cytokine that potentially inhibits alloresponse in MLR [44]. Furthermore, a previous study showed that amnion epithelial cells also expressed inhibitory cytokine such as IL-4 in protein and mRNA [45]. An obvious assumption therefore is that human AM-associated suppressor function could be due to the production of those inhibitory cytokines. To assess this possibility, we investigated the production by human AM of selected human cytokines thought to possess immune-suppressive effects, such as IL-4, IL-10, TGF-β1. However, we could not detect IL-4, IL-10 or TGF-β1 in the culture supernatant of MLR with human AM in transwells. Although those findings suggest that human AM in MLR culture did not produce the known inhibitory cytokines, including IL-4, IL-10 and TGF-β, additional confirmation is required as the lack of such inhibitory cytokine synthesis at the mRNA level. Further, it is important to examine whether stimulated human AM is capable of producing these inhibitory cytokines.

In the present study, we could not determine the AM soluble factor that suppresses allo-reactive T cells. In addition to the possibility of inhibitory cytokines, PGE_2, which can inhibit IL-2 synthesis, may have immunosuppressive ability [46–48]. A previous study has indicated that human amnion cells produce PGE_2 which can be enhanced by the presence of granulocyte supernatants. It has been also demonstrated that HLA-G inhibits the allogenic proliferative response [49], and that soluble HLA-G is present in amniotic fluids [50]. Furthermore, human fetal membrane expresses FasL, by which the fetus is afforded protection against the cytolytic actions of lymphocytes from the mother [51–54]. Thus, it is possible that the inhibitory effect of human AM could be explained by the presence of PGE_2, HLA-G and FasL, in addition to inhibitory cytokines. In addition, we have to consider that these inhibitory factors could be induced by exposure of human AM to soluble factors derived from lymphocytes in MLR culture. To formally assume that human AM produces a new inhibitory molecule for suppression of allo-graft rejection in ALT, we must carefully perform a series of additional experiments. These lines of study are under investigation in our laboratory.

In summary, our present data demonstrate that human AM is capable of inhibiting allo-reactive T cell response including cell division, proliferation and Th1/Th2 cytokine synthesis *in vitro*. Further, our findings suggest the interesting possibility that human AM may secrete an undefined inhibitory factor for the suppression of allogenic response. This inhibitory effect of human AM likely contributes to the success of the ALT-AMT combination in ocular surgery.

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Characterization of protective immune responses induced by nasal influenza vaccine containing mutant cholera toxin as a safe adjuvant (CT112K)

Izumi Watanabe [a,b], Yukari Hagiwara [a,c], Shin-etsu Kadowaki [a], Tomoki Yoshikawa [a], Katsuhiro Komase [c], Chikara Aizawa [c], Hiroshi Kiyono [d], Yoshifumi Takeda [a], Jerry R. McGhee [e], Joe Chiba [b], Tetsutaro Sata [a], Takeshi Kurata [a], Shin-ichi Tamura [a,*]

[a] *Department of Pathology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*
[b] *Department of Biological Science and Technology, Science University of Tokyo, 2641 Yamazaki, Noda, Chiba 278-8510, Japan*
[c] *Research Center for Biologicals, Kitasato Institute, 6-111 Arai, Kitamoto-shi, Saitama 364-0026, Japan*
[d] *Department of Mucosal Immunology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan*
[e] *Department of Microbiology, University of Alabama at Birmingham, BBRB 761, Birmingham, AL 35294-2170, USA*

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Abstract

Immune responses induced by a nasal influenza vaccine with a mutant cholera toxin (CT112K), known to be a safe adjuvant, were characterized in BALB/c mice to confirm the most suitable regimen of this vaccine for humans. Mice received a primary intranasal administration of the adjuvant (0.1 μg)-combined PR8 vaccine (0.1 μg) and a secondary administration of the PR8 vaccine alone (0.1 μg) 4 weeks later. Two weeks after the secondary immunization, the mice were infected with a nonlethal or a lethal dose of PR8 viruses. Nasal and lung wash virus titers 1 or 3 days after infection indicated that complete protection could be provided by secondary immune responses, which had an immediate effect of preventing infection 2 weeks after the secondary immunization. In this two-dose regimen, high levels of secondary IgA, IgG and IgM antibody-forming cell (AFC) responses were induced in the nasal-associated lymphoid tissue and the spleen. In parallel with the AFC responses, high levels of nasal wash anti-PR8 HA IgA, and lung and serum IgG antibody (Ab) responses were induced 2 weeks after the secondary immunization. The two-dose regimen also induced accelerated delayed-type hypersensitivity responses, which exhibited almost the same peak height as that in the case of the primary response. In addition, the two-dose regimen induced a low memory cell activity of cytotoxic T lymphocytes, detected by in vitro culture of spleen cells. Thus, the immediate effect of preventing infection was mainly provided by the secondary Ab responses. Moreover, the levels of nasal wash IgA Abs correlated well with cross-protection against infection with variant viruses in the upper respiratory tract (RT). These results suggest that the major protective factors among Ab and T cell-mediated immune responses, which are induced by the two-dose regimen using CT112K-combined vaccines, are the cross-reactive IgA Abs in the upper RT and the less cross-reactive IgG Abs in the lower RT, and that the two-dose regimen is a suitable vaccination condition for humans.

Keywords: Influenza; Nasal vaccine; Mutant cholera toxin; IgA

1. Introduction

The respiratory tract (RT) mucosa is the site of either infection by influenza viruses or defense against the virus infection in the host animals [1]. Influenza viruses infect host cells by binding to the receptor with a viral surface glycoprotein, hemagglutinin (HA), and are released from the infected host cells by the action of another surface glycoprotein, neuraminidase (NA), after replication. The viruses cause an annual epidemic of a serious respiratory infectious disease (influenza) by altering the antigenic properties of the HA and NA glycoproteins. To prevent influenza, the immune responses, in which multiple components from both innate immune responses and adaptive immune responses are involved, are induced by the virus infection [2–4]. If influenza viruses can breach the early lines of innate immunity, they are detected and eliminated by adaptive immune

* Corresponding author. Present address: Laboratory of Prevention of Viral Diseases (Research Foundation for Microbial Diseases of Osaka University), Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. Tel.: +81-6-6879-4251; fax: +81-6-6879-4250.
E-mail addresses: stamura@biken.osaka-u.ac.jp, stamura@nih.go.jp (S.-i. Tamura).

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responses, in which IgM, IgG and IgA antibodies (Abs), $CD4^+$ Th1 cells, and $CD8^+$ T cells are involved [2–6]. Among the immune responses induced by the virus infection, secretory IgA (S-IgA) Abs to the HA glycoprotein are major effector molecules that provide cross-protection against variant virus infection [7–11]. The S-IgA has an overall higher avidity for influenza virus than serum IgG, which is derived from the polymeric nature of S-IgA Abs [8,12]. The S-IgA Abs are dimeric IgA Abs, which are produced by IgA Ab-forming cells (AFCs) in the lamina propria and secreted actively to the mucosal surface across mucosal epithelial cells [13,14]. AFCs are first induced as precursors of IgA-AFCs in the nasal-associated lymphoid tissue (NALT) soon after infection and disseminated to the lamina propria underneath the mucosal epithelium via general circulation [15–17]. Thus, in parallel with the development of AFCs, the S-IgA Abs appear in the nasal mucus [17].

To control influenza, currently used inactivated vaccines are administered parenterally. The parenterally administered inactivated vaccines mainly induce serum IgG Abs to HA, which protect against homologous virus infection. Thus, the protective efficacy of the current vaccines is low against an epidemic of heterologous viruses [1]. On the other hand, the findings, which were described in the preceding paragraph, that the S-IgA Abs in the RT mucosa can provide cross-protection against variant virus infection suggests that the protective efficacy of the current vaccines will be improved by the development of methods of inducing the S-IgA Abs [7–12]. In this regard, we attempted to induce the S-IgA Abs in the RT by intranasal immunization of an inactivated vaccine containing cholera toxin (CT) [18].

Cholera toxin (CT), produced by *Vibrio cholerae*, and *Escherichia coli* heat-labile enterotoxin (LT) whose sequence shows 80% similarity with that of CT, are strong mucosal adjuvants that enhance immune responses to mucosally co-administered bystander antigens [19–21]. Needless to say, both toxins are the causative agents of cholera and traveler's diarrhea. These toxins consist of a binding region of five B subunits, into which the active toxic component, the A subunit, is inserted. The toxins bind to a GM1 ganglioside via the B subunits on the cell membrane. This enables translocation of the A subunit across the membrane into the cell, where the A subunit is cleaved by intracellular protease into A1 and A2 components. The A1 component is an ADP-ribosylating enzyme that binds NAD and transfers the ADP-ribose moiety to a target GTP-binding protein (Gs), resulting in intracellular accumulation of cAMP and cell intoxication [22–24].

The use of these toxins as adjuvants of mucosal vaccine in humans is not feasible owing to its toxicity. One approach to reducing the toxicity of the toxins is to use the B subunits (CTB or LTB) containing a trace amount of the toxin (CTB* or LTB*), because the B subunit and a trace amount of the holotoxin act synergistically as an adjuvant of vaccines that induce mucosal immune responses [25–27]. We have demonstrated that the intranasal administration of the CTB*-combined influenza vaccine provided cross-protection against variant strains within one subtype of A type virus, by inducing cross-reacting anti-HA IgA Abs in the upper RT [28–31]. We have also demonstrated that the cross-protection was provided effectively in mice that received a primary CTB*-combined vaccine and a secondary vaccine alone 4 weeks later [28].

A dose of about 0.1 μg of adjuvant or vaccine per mouse used in a two-dose regimen, was close to the minimal effective dose at which an adjuvant of nasal influenza vaccine functions effectively in BALB/c mice [28]. These preclinical studies suggest that the intranasal spraying of a small volume of an adjuvant-combined trivalent vaccine in a two-dose regimen provided cross-protection against all epidemic strains. The field trials performed on humans under these conditions showed that the adjuvant-combined vaccine induced a significantly high level of anti-HA secretory IgA and systemic IgG Abs as compared with the vaccine alone, suggesting that it was effective in preventing influenza [32,33]. The dose of LTB* or the vaccine used in the field trials was 100 μg per person, corresponding to about 0.1 μg per mouse. No serious adverse effects associated with the adjuvant-combined nasal influenza vaccine were observed omit [33,34], although the possibility that the toxicity of CTB on the brain may be manifested by the binding of CTB to the olfactory nerves in the nasal area is indicated [35].

Moreover, adjuvants safer than CTB* or LTB*, in which the mutated toxins devoid of toxicity but retaining its adjuvant action, were developed and their use as adjuvants for nasal influenza vaccine has been attempted [36–38]. We have demonstrated that the mutant CT112K (Glu-to-Lys substitution at amino acid position 112 of the A1 subunit) seems to be the most effective and safest adjuvant among several CT mutants examined [39]. The mutant CT112K showed a relatively high adjuvant activity, which is slightly less than that of native CT when the adjuvant activity was analyzed based on the ability of immunized mice to induce anti-HA Ab responses and to provide protection against infection. The mutant is also less toxic than native CT when the toxicity was analyzed using Y1 adrenal cells in vitro, is less allergenic than native CT when the allergenicity was analyzed based on the ability of immunized mice to induce anti-CTB IgE Ab responses, and is obtained at relatively high yields in a bacterial culture. With these as a background, Ab and T cell-mediated immune responses induced by the nasal CT112K-combined influenza vaccine remain to be sufficiently characterized. The characterization will be required to confirm the conditions under which this vaccine is suitable for humans.

In the present study, immune responses induced by the nasal CT112K-combined influenza vaccine were characterized in BALB/c mice, which received a primary administration of the adjuvant (0.1 μg)-combined vaccine (0.1 μg) and a secondary administration of the vaccine alone (0.1 μg) 4 weeks later. In this two-dose regimen, anti-PR8 vaccine AFC responses, anti-PR8 HA Ab responses, delayed-type

hypersensitivity (DTH) responses to the PR8 vaccine and cytotoxic T cell responses to PR8 virus-infected target cells were assayed. The results suggested that the major protective factor among Ab and T cell- mediated immune responses induced by the two-dose regimen using CT112K-combined vaccine are IgA and IgG Ab responses.

2. Materials and methods

2.1. Mice

Female BALB/c mice (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu-shi, Japan), aged 6–8 weeks at the time of immunization, were used in all experiments.

2.2. Preparation of CT, CTB and CT112K

Commercially available CT and CTB were obtained from Sigma (St. Louis, MO). CTB* was prepared by adding 0.2% of CT (Sigma) to CTB (Sigma). CT112K was prepared as described previously [39]. Briefly, a 5.4 kbp *Eco*RI/*Pst*I DNA fragment including the CT gene from *Vibrio cholerae* OGAWA NIH 412 was cloned into plasmid pUC119. This clone, which is capable of producing recombinant CT, was designated as pUCCT10. CT112K (Glu-to-Lys substitution at amino acid position 112) was constructed by site-directed mutagenesis, in which amino acid substitutions were introduced into pUCCT10 by the PCR method using specific primers. Recombinant CT112K was purified according to the method described by Uesaka et al. [40]. Briefly, the bacteria were harvested and lysed with a sonicator (Biorupter, Tousoudenki Co. Ltd., Japan). The crude lysate was then applied to immobilized D-galactose columns and eluted with galactose. The yields of CT112K per liter was about 70 μg. The purified CT112K contained less than 0.05 ng/μg LPS in the Limulus test [41].

2.3. HA vaccines and influenza viruses

HA vaccines (split-product virus vaccines) were prepared from influenza viruses, A/PR/8/34 (PR8, H1N1), A/Yamagata/120/86 (A/Yamagata, H1N1), A/Beijing/262/95 (A/Beijing, H1N1) and B/Ibaraki/2/85 (B/Ibaraki) by the method of Davenport et al. [42] at the Kitasato Institute, Saitama-Ken. These viruses, except for PR8, have been used recently to prepare vaccines in Japan. They are immunologically different from one another [30]. The PR8 virus-immunized mice had serum hemagglutination inhibition (HI) Abs that are cross-reactive to A/Yamagata (H1N1) and A/Beijing (H1N1) vaccines, at titers of 1/128 and lower than 1/128, respectively. The PR8 virus-immunized mice had no serum HI Abs that are cross-reactive to B/Ibaraki vaccines (titers less than 1/128). The PR8 virus was passaged 148 times in the ferret, 596 times in the mouse, and 73 times in 10-day chicken fertile eggs.

2.4. Immunization

Groups of five mice for each experiment were anesthetized by intraperitoneal injection of amobarbital sodium (0.25 ml of 1 μg/ml solution). Then, they were immunized primarily by dropping 1 μl of PBS containing a CTB112K (0.1 μg/2 μl)-combined HA vaccine (0.1 μg/2 μl) into each nostril. Four weeks later, they were boosted in a similar manner with the HA vaccine (0.1 μg/2 μl) alone.

2.5. Infection

A mouse-adapted PR8 virus was used for infection. Two conditions for virus infection regarding the site of infection were used according to a modified procedure of Yetter and coworkers [5,17,43]. Under one of these conditions, each mouse was anesthetized and then infected by intranasally dropping 20 μl of PBS containing a virus suspension with $10^{4.1}$ EID_{50} per mouse. This procedure induced a total respiratory tract infection that caused virus shedding from the nose and the lung, and led to death from viral pneumonia about 7 days later. The virus inoculum was about $20 \times LD_{50}$, when LD_{50} was determined by the total respiratory infection method. The nasal and lung wash virus titers on 3 days after infection were used as indices of protection in the upper and the lower respiratory tracts of immunized mice, respectively. Under another infection condition, anesthetized mice were infected by intranasally dropping 1 μl of PBS containing a virus suspension with $10^{4.1}$ EID_{50} ($20 \times LD_{50}$) into each nostril. The nose-restricted volume (2 μl) of the virus suspension induced nose-localized infection, which was initiated in the nasal mucosa and spread to the lungs in 3–7 days but was not lethal. The nasal wash virus titers, the peaks of which were attained 3 days after infection, were used as indices of protection in the upper RT of immunized mice.

2.6. Nasal wash, lung wash and serum specimens

The mice were anesthetized with chloroform and then bled from the heart with a syringe. Serum was separated from blood by centrifugation and used for Ab titration. After bleeding, the mice were incised ventrally along the median line from the xiphoid process to the chin. The trachea and lungs were removed and washed twice by injecting a total of 2 ml of PBS containing 0.1% BSA [5]. The mice were then decapitated and the lower jaws excised. A hypodermic needle was inserted into the posterior opening of the nasopharynx and 1 ml of PBS containing 0.1% BSA was injected three times to collect the nasal wash. The nasal and lung washes were used for Ab and virus titrations after removing cellular debris by centrifugation. The physical manipulation of nose and lung to collect secretions at the luminal surface caused blood leakage from the lamina propria. Blood

contamination of the nasal and the lung wash was estimated by counting the red blood cells and comparing the count with that of blood (about 8×10^9 cells/ml), for nasal and lung wash specimens from 10 mice. The blood contaminations of nasal and bronchoalveolar (lung) wash specimens were 0.003 ± 0.002 and 0.000 ± 0.000 μl (mean ± S.D.), respectively.

2.7. Virus titrations

Serial 10-fold dilutions of the nasal wash were prepared, and a 0.2 ml aliquot of each dilution was inoculated into Madin–Darby canine kidney (MDCK) cells in a six-well plate. After 1 h of adsorption, each well in the plate was overlaid with 2 ml of agar medium according to Tobita and coworkers [44,45]. After 2 days of incubation in a CO_2 incubator, the plaques were counted. The virus titer was expressed in PFU/ml. The virus titer of each experimental group was presented as the mean ± S.D. of the virus titers per ml of specimens from five mice in each group.

2.8. Nasal-associated lymphoid tissues (NALTs) and spleens

Mice were sacrificed on various days after intranasal immunization, and their NALTs and spleens were removed, as described previously [16]. Cell suspensions from the NALTs and spleens were prepared, as described previously [17].

2.9. Enzyme-linked immunospot (ELISPOT) assay

Influenza virus-specific Ab-forming cells (AFCs) were enumerated by an ELISPOT assay, as described previously [46,47]. Briefly, a 96-well plate with a nitrocellulose base (Millititer HA; Millipore) was coated with the PR8 HA vaccine, then blocked with FCS in PBS. Lymphocytes were suspended in a medium containing FCS and this suspension was diluted serially. The dilutions were added to each well and incubated for 4 h. The plate was washed with PBS–Tween and incubated with goat anti-mouse IgA (α-chain-specific) (Life Technologies) or goat anti-mouse IgG (γ-chain-specific) (Southern Biotechnology Associates) conjugated with alkaline phosphatase. Spots representing single Ab-secreting cells were developed with the substrate. The spots were counted under a dissecting microscope.

2.10. ELISA

The levels of IgA and IgG Abs against HA molecules purified from PR8 viruses were determined by ELISA as described previously [30]. Briefly, ELISA was carried out sequentially in the solid phase (EIA plate, Coster, Cambridge, MA) with a ladder of reagents consisting of the following: first, HA molecules purified from the PR8 virus according to the procedure of Phelan et al. [48]; second, nasal wash, bronchoalveolar wash or serum; third, goat anti-mouse IgA Ab (α-chain-specific) (Amersham) or goat anti-mouse IgG Ab (γ-chain-specific) (Amersham) conjugated with biotin; fourth, streptavidine conjugated with alkaline phosphatase (GIBCO BRL); and finally, *p*-nitrophenyl-phosphate. The chromogen produced was measured at an absorbance of 410 nm using a SJ eia Autoreader (model er-8000, Sanko Junyaku Co. Ltd., Tokyo, Japan). The purified HA-specific IgA or HA-specific monoclonal IgG at 100 ng/ml was used as a standard. The Ab concentration of an unknown specimen was determined from the standard regression curve constructed for each assay using the programmed SJ eia Autoreader. The chromogen produced was measured at an absorbance of 410 nm using an ELISA reader.

2.11. DTH assay

The DTH reaction was elicited by injecting a HA vaccine (5 μg/ml) in PBS in a 25 μl volume into the right hind footpad of each animal and injecting 25 μl of PBS into the left hind footpad as a control. The increase in thickness of the footpad (footpad swelling) was measured 24 h later as a measure of the DTH reaction with a dial thickness gauge (Peacock G; Ozaki Seisakusho, Tokyo, Japan), as described previously [5].

2.12. CTL assays

Splenic CTL activity was determined following a 5-day in vitro stimulation with PR8-infected autologous splenocytes, according to the methods of Bennink and coworkers [49,50] and our method [5,51]. Briefly, spleen cells ($(3–5) \times 10^6$ cells/ml) were suspended in Iscove's modified Dulbecco's medium containing 10% fetal calf serum (FCS), 5×10^{-5} M mercaptoethanol and 60 μg/ml of kanamycin sulfate (Iscove's medium). One-third of the spleen cell suspension was incubated for 1 h with $10^{7.5}$ EID_{50} of the PR8 virus and the PR8-infected splenocytes were then mixed with the remaining two-thirds of the spleen cell suspension. The mixture were incubated for 5 days in 5% CO_2 at 37 °C. At the end of the incubation period, the cells were harvested, counted and re-suspended as effector cell populations of 2×10^6, 6×10^5, 2×10^5 and 6×10^4 cells/ml. P815 cells (DBA/2 mastocytoma cells, 3×10^6) (H-2^d), which were target cells, were incubated with $10^{7.5}$ EID_{50} of the PR8 virus and 3.7 MBq of $Na_2{}^{51}CrO_4$ (NEN Life Science Products Inc., Boston, MA) at 37 °C for 1 h. The PR8-infected and ^{51}Cr-labeled P815 cells were re-suspended at 1×10^6 cells/ml, and 100 μl of the cell suspension was added to a 96-well round-bottomed microtiter plate containing triplicate100 μl samples of serially diluted effector cells. The microtiter plate was incubated at 37 °C for 6 h and centrifuged. The supernatants (100 μl) were removed from each well and the level of released radioactivity was determined using a gamma counter. Specific lysis was calculated from the ^{51}Cr release in counts per minute (cpm) using the formula: %specific lysis = [(experimental cpm − spontaneous cpm)/(maximal release cpm − sponta-