Rhinology, 43, 190-198, 2005

Mucosal immunity of nasopharynx: an experimental study in TCR-transgenic (OVA23-3) mice*

Takaya Yamada[1,2], Shingo Kataoka[1], Keiko Ogasawara[1], Ryotaro Ishimitsu[1], Kazuhiro Hashigucci[3], Tatsuo Suzuki[2], Hideyuki Kawauchi[1]

[1] Department of Otolaryngology, Shimane University, Izumo City, Japan
[2] Biomedical Laboratory, Kitasato Institute Hospital, Tokyo, Japan
[3] Otolaryngology Clinic, Kitasato Institute Hospital, Tokyo, Japan

SUMMARY

The ideal vaccine therapy has been warranted for activation of the mucosal immune response in the upper respiratory tract against various types of microbial infection. However, the precise study in regard to the mucosal route of vaccine administration and its mechanism of action remains to be further investigated. Therefore, to better understand the exact mechanism of nasopharyngeal mucosal immunology, from T-cell aspects, the antigen-specific antibody response was investigated in T cell receptor transgenic (OVA23-3) mice (Tg-mice) and wild type BALB/c mice, in comparison, which were stimulated with repeated nasal antigen challenges of ovalbumin (OVA) together with cholera toxin (CT) or OVA alone.

OVA-specific IgA and IgG antibodies were not detected in nasal washings of BALB/c mice when these mice were intranasally stimulated with OVA alone. But they were detected in those of BALB/c mice stimulated with OVA and CT, as we have already reported. Interestingly, OVA-specific IgA and IgG antibodies were significantly higher in nasal washings of Tg-mice stimulated with OVA and CT or OVA alone rather than those of BALB/c mice stimulated with OVA and CT.

In line with data of the antibody response, OVA-specific IgA and IgG antibody-producing cells significantly increased in number in nasal passage (NP), nasopharyngeal-associated lymphoreticular tissue (NALT), cervical lymph node (CLN), and spleen (SP) of these mice.

In nasal washings of Tg-mice, interferon (IFN)-γ and interleukin (IL)-4 was detected even with a small amount of antigen. To see the cytokine profile of NALT, NP, CLN, and SP of these mice, various cytokine concentrations were measured in supernatants of these cells cultured in vitro with OVA. As a result, IFN-γ was detected at significantly higher levels in culture supernatants of lymphocytes sampled from NP, CLN, SP as well as NALT of mice having increased antibody titers in nasal washings. On the other hand, Th2 type cytokines such as IL-4, IL-6 and IL-13 were efficiently detected in culture supernatants of NP, CLN, and SP cells from Tg-mice mice, but not in those from NALT cells of those mice.

All these data taken together indicate that helper T cells recruited into nasal mucosa and locally activated in an antigen-specific fashion, as well as NALT T cells, are essential for mounting local antigen-specific antibody responses.

Key words: mucosal immnity, IgA, NALT, ovalbumin, TCR-transgenic (OVA23-3) mice, cytokine

INTRODUCTION

Thanks to the development of molecular biological techniques, the nasal mucosa has become a focus of studies on mucosal immune responses in order to confirm theoretical bases and pursue its clinical application for the vaccination therapy against microbial infections and hyposensitization therapy for nasal allergy. Despite the recent emphasis on elucidation of the molecular and cellular aspects of the mucosal immune system, very little information is yet available in regard to the nasopharyngeal mucosal immune system in comparison with those in the gastrointestinal tract.

Secretory IgA antibodies act to expel bacterial and viral

*Received for publication: August 18, 2003; accepted: December 29, 2004

pathogens from the body, bacterial toxins and other potentially harmful molecules, mediate humoral immunity and antibody-dependent, cell-mediated cytotoxicity (in cooperation with macrophages, lymphocytes and eosinophils), and prevent the utilization of growth factors by bacterial pathogens in the mucosal environment [1].

In mucosal sites, various kinds of immunocompetent cells (e.g., epithelial cells, T cells, B cells, dendritic cells and macrophages) form a unique mucosal immune network, and they contribute to the induction of specific and protective immunity against foreign antigens. Antigen-specific immunoglobulin A (IgA) produced in nasopharyngeal mucosa and secreted through the epithelial linings as secretory IgA and homing of IgA-precursor cells and regulatory T cells into nasopharyngeal mucosa among common mucosal immune system (CMIS) is the most important element in the mucosal specific defense system [2-4].

In humans, the pharynx is guarded by Waldeyer's ring tonsils, which is considered as an equivalent to the nasopharyngeal-associated lymphoreticular tissue (NALT) in rodents containing B and T cells, dendritic cells, and an absorptive crypt epithelium including M cells, befitting their role as an immune inductive site [5]. A functionally equivalent tissue as NALT has been reported in rodents (e.g., mice and rats)[6-8]. NALT in rodents was found to be bilateral strips of non-encapsulated lymphoid tissue underlying the epithelium on the ventral aspects of nasopharyngeal duct [8, 9]. The role of NALT is considered to initiate an antigen-specific immune response after nasal immunization as an inductive site of IgA response, which is well characterized as Peyer's patches in the gastrointestinal tract [10]. Alternatively, the nasal passage (NP) can be considered the same as the intestinal lamina propria, an example of an IgA effecter site.

Thus, for the last decade, we have been studying local immune responses in the nasopharynx and tubotympanum by animal experiments and employing human specimens such as nasal mucosae, paranasal sinus mucosae, and tonsillar tissues. In our series of animal experiments, we have already demonstrated that antigen-specific mucosal immunity (IgA response) is provoked by nasal protein antigen challenges together with cholera toxin (CT) and that T cell subsets as well as antibody-producing B cells are essential to mount a local antibody production [11,12].

Therefore, in this study, to further confirm the exact role of T cells in the induction of IgA responses in nasal mucosae, immunological experiments were carried out in T-cell receptor transgenic (OVA23-3) mice (Tg-mice) as well as wild type BALB/c mice, when those were stimulated with repeated nasal antigen challenges of chicken ovalbumin (OVA) together with CT or without CT. In these experiments, antigen-specific IgA and IgG antibody titers in nasal washings and the frequency of antibody-producing B cells in NALT, NP, cervical lymph node (CLN) and spleen (SP) were respectively determined by ELISA and ELISPOT assays. Furthermore we were interested to see the cytokine profiles of T cells; interferon (IFN)- γ and interleukin (IL)-4 levels were measured in nasal washings and various Th1 and Th2 type cytokine productions were respectively measured in culture supernatants of NALT, NP, CLN and SP cells.

MATERIALS AND METHODS

Animals

Tg-mice were kindly supplied by Professor Habu (Tokai University, Isehara, Japan). These mice express T cell receptor-α β chain genes, derived from the chicken ovalbumin (OVA)-specific I-A^d-restricted $CD4^+CD8^-$ T helper cell clone 7-3-7 [13]. BALB/c male mice were purchased from the animal breeder (Japan CLEA, Tokyo, Japan). All mice used in this study were 6-9 weeks of age.

Immunization

Mice were intranasally immunized on days 0, 2, 4, 6, 8, 10 and 12 with 2μL of phosphate-buffered saline (PBS) containing a mixture of 100μg ovalbumin (OVA)(Sigma Chemical, St. Louis, USA) and 1μg of cholera toxin (CT) (Sigma Chemical) as a mucosal adjuvant, or OVA alone.

OVA-specific antibody assay

Nasal washings were collected at 0, 1, 2, 3, 4, 5 and 6 weeks after the final challenge of the antigens. After blood was collected from the femoral artery under anaesthesia with ether, and then perfused transcardially with 5 mL of cold PBS to prevent blood contamination into nasal washings. Nasal washings were collected by washing the nasal cavity with 200μL of cold PBS according to a method previously reported [14].

OVA-specific IgA and IgG antibody titers in nasal washings and serum samples were determined by enzyme-linked immunosorbent assay (ELISA) as described previously Briefly, 96-well plates (ICN Biomedicals, USA) were coated with an optimal concentration of OVA (100μL of a 10μg/ml concentration of OVA) in 0.1 M of carbonate buffer. The plates were incubated overnight at 4°C in a humidified atmosphere and washed three times with 0.05% PBS-Tween20 solution (PBS-Tw). Wells were blocked with 200μL of PBS containing 1% bovine serum albumin (BSA; Sigma Chemical) for 1h at 37°C. After extensive washings, serial dilutions of the samples were added and incubated for 1h at 37°C. After incubation and washing, 100μL of a 1:500-diluted biotinylated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc., USA) or 100μL of a 1:250-diluted biotinylated goat anti-mouse IgA (Zymed Laboratories, Inc., San Francisco, USA) was added to the wells. After incubation for 1h at room temperature and washed five times, plates were further incubated for 20 min at room temperature. A 100μL/well volume of ophenylendiamine solution (1.5 mg/ ml with 1% H_2O_2) was added as detection solution to each well. Then, the colorimetiric reaction was read an OD of 490nm on a micro-ELISA reader.

Isolation of mononuclear cells

NALT, NP, CLN and SP were dissected out aseptically under the microscope and minced in Hank's balanced salt solution (HBSS) and these cells were prepared as cell suspensions for further processing. Mononuclear cells were isolated using a discontinuous Lympholyte-M gradient (Cedarlane, Ontario, Canada). Cells from different organs were resuspended in RPMI-1640 medium supplemented with glutamine, 2-mercaptoethanol, 10% fetal calf serum and penicillin-streptomycin, and used for enumeration of immunoglobulin-producing cells and *in vitro* culture for a measurement of cytokine production.

Enumeration of immunoglobulin-producing cells

For an enumeration of OVA-specific immunoglobulin-producing cells, the number of OVA-specific IgA-producing and IgG-producing cells in the NALT, NP, CLN, and SP were respectively determined by enzyme-linked immunospot (ELISPOT) assay as described previously. Briefly, 96-well filtration plates with a nitrocellulose base (Millititer HA; Millipore Corp., Bedford, USA) were coated with 1μg of OVA per ml and incubated overnight at 4°C. The plates were washed three times with PBS and then blocked with RPMI-1640 medium for 1 h. The blocking medium was removed, and various concentrations of test cells in RPMI-1640 medium were added and cultured at 37°C in air with 5% CO_2 and 95% humidity for 4 h. After incubation, plates were washed three times with PBS and then one time with PBS-Tw. After incubation and washing, 100μL of a 1:500-diluted biotin-labeled goat anti-mouse IgA (Southern Biotechnology Associates, Inc., Birmingham, USA) or 100μL of a 1:1000-diluted biotin-labeled goat anti-mouse IgG (Zymed Laboratories)-specific antibodies was added to all wells. After overnight incubation at 4°C, the plates were washed five times with PBS-Tw, and an aliquot of 100μL of avidin-perozidase (Zymed Laboratories), diluted 1:1000 in PBS-Tw, was added to each well. The plates were incubated in the dark at room temperature for 1 h, and then the spots were developed for 20 minutes at room temperature with 100μL of 0.1 mM 3-amino-9-ethylcalbazole (Sigma Chemical) in 0.1 M sodium acetate buffer containing 0.05% H_2O_2, after washing with water. Thereafter, red-brown-colored spots were counted as OVA-specific-antibody-forming cells, and expressed as the mean number of SFC per 10^6 cells, triplicate determination.

Analysis of cytokine production in NP, NALT, CLN, and SP cells and measurement of it in nasal washing

Lymphocytes obtained from NALT, NP, CLN and SP ($2.5x10^5$) were cultured with 200μg of OVA for 48 h at 37°C in 200μL of RPMI-1640 medium in flat-bottomed 96-well culture plates (Corning, Incorporated, NY, USA). Thereafter microplates were centrifuged and culture supernatants were harvested and frozen at -80°C until measurement.

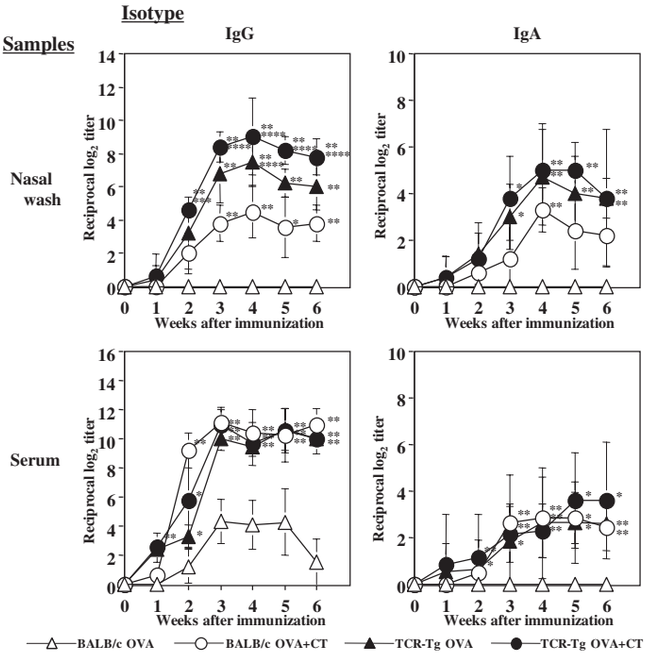
Commercial ELISA kits were respectively used to measure levels of IFN-γ, IL-4 and IL-6 (Genzyme Techne Corporation, Minneapolis, USA), IL-5 (Pharmingen, San Diego, USA), IL-13 (Quantikine, Murine, USA) in culture supernatants and nasal washings.

RESULTS

Intranasal immunization induces OVA-specific antibody response in nasal washings of Tg-mice without CT

As a first step, we examined OVA-specific antibody response in nasal mucosae and sera of wild type BALB/c mice and Tg-mice, following repeated intranasal challenges of OVA with or without CT. As a result, OVA-specific IgA and IgG antibodies significantly increased in nasal washings of BALB/c and Tg-mice stimulated with OVA together with CT (OVA+CT-immunized group) and even in those of Tg-mice stimulated with OVA without CT (OVA-immunized group) (Figure 1).

Figure 1. Kinetics of IgG and IgA anti-OVA antibody responses in nasal wash (a) or serum (b) from BALB/c (open symbols) or TCR-transgenic (closed symbols) mice. Mice were immunized intranasally with OVA (100μg) with CT (1μg)(circle) or OVA alone (triangle) on days 0, 2, 4, 6, 8, 10 and 12. Each point represents the mean ± SEM of the antibody titers from ten mice per groups. * = $p<0.05$; ** = $p<0.01$ vs OVA alone immunized BALB/c mice. *** = $p<0.05$, **** = $p<0.01$ vs OVA+CT-immunized BALB/c mice.



These antibody responses peaked 2 weeks after the final intranasal OVA challenge, although antibody responses seemed to occur with a higher amount in Tg-mice rather than wild type BALB/c mice (Figure 1). In nasal washings of the Tg-mice stimulated with OVA together with or without CT, IgG and IgA titers were firstly detected in the first week, peaked in the fourth to fifth week, and then declined. OVA-specific IgA and IgG antibody titers in nasal washings in weeks 2 to 6 were signifi-

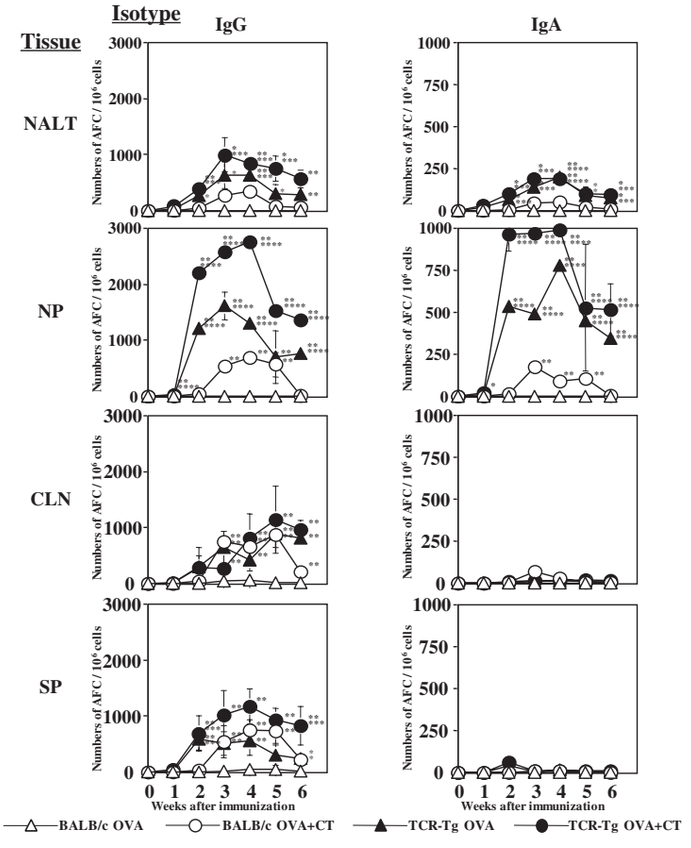
cantly higher ($p<0.05$) in the Tg-mice stimulated with OVA with or without CT, in comparison with those in BALB/c mice stimulated with OVA.

OVA-specific IgG and IgA antibody titers in sera were also higher in the BALB/c mice stimulated with OVA and CT, and in Tg-mice stimulated with OVA together with or without CT at each interval after intranasal challenge.

Nasal immunization induces OVA-specific IgG and IgA producing B cells in mucosal effector tissues.

The specific IgG and IgA producing B cells in NALT and NP cells significantly increased in number in BALB/c mice stimulated with OVA and CT, and in Tg-mice stimulated with OVA with or without CT. However, IgA producing B cells were not significantly detected in CLN and SP, but IgG producing B cells were detected in CLN and SP (Figure 2). The number of IgG and IgA producing B cells in NALT and NP seemed to peak at two weeks after the final intranasal challenge and declined afterwards. Interestingly, in BALB/c mice stimulated with OVA and CT, and Tg-mice stimulated with OVA with or without CT, IgG and IgA antibody-producing B cells in NALT were detected in smaller numbers than those in NP.

Figure 2. Kinetics of IgG and IgA OVA-specific antibody-producing cells in the NALT, NP, CLN and SP from BALB/c (open symbols) or TCR-transgenic (closed symbols) mice. OVA-specific antibody-producing cells were determined by ELISPOT assay. Each value represents the mean ± SD in duplicate (NALT), triplicate (NP) or sixth (CLN and SP) ELISPOT assays of pooled cell suspensions from five mice. * = $p<0.05$; ** = $p<0.01$ vs OVA alone immunized BALB/c mice. *** = $p<0.05$; **** = $p<0.01$ vs OVA+CT-immunized BALB/c mice.



Cytokine levels in nasal washings

Th1 and Th2 types of cytokine levels were measured in the nasal washings sampled from each group of mice (Table 1). The amount of cytokines was not measurable in nasal washings obtained from BALB/c mice stimulated with OVA with or without CT. On the other hand, IFN-γ and IL-4 was detected in nasal washings of Tg-mice stimulated with OVA with or without CT and seemed to be peaking in the second week. No other cytokines were measurable in nasal washings of these mice. IL-6 and IL-13 were detected in nasal washings of Tg-mice stimulated with OVA and CT only at two weeks after starting the intranasal OVA challenge.

Analysis of cytokine production of NALT, NP, CLN, and SP cells

To elucidate the role of T cells in mucosal IgA and IgG response, the cytokine profiles of T cells in NALT, NP, CLN and SP cells were examined. IFN-γ production, and on the other hand IL-4, IL-5, IL-6, and IL-13 production, are respectively noted as a representative of Th1 type and Th2 type cytokines. Those cytokine levels were measured in the culture supernatants harvested from wells containing NALT, NP, CLN and SP cells derived from each group of mice (Tables 2, 3, 4, 5 and 6).

No significant Th1 or Th2 type cytokine production was detected in the culture supernatants of lymphocytes obtained from various tissues of BALB/c mice stimulated with OVA alone. The lymphocytes obtained from NALT, NP, CLN, and SP of Tg-mice stimulated with OVA together with or without CT, released the highest levels of Th1 type (IFN-γ) and Th2 type (IL-4, IL-5, IL-6, and IL-13) cytokines into the culture supernatants at each interval after intranasal immunization and these cytokine productions peaked at 2 weeks. The lymphocytes obtained from NALT, NP, CLN, and SP of BALB/c mice stimulated with OVA and CT, showed significant IFN-γ production at each interval (1-3 week).

In regard to the Th2 cytokines, IL-4, IL-6, IL-13 productions were transiently observed in culture supernatants of NP and CLN of BALB/c mice stimulated with OVA and CT. But Th2 type cytokine production was not detected in the culture supernatants of NALT lymphocytes obtained from BALB/c or Tg-mice stimulated with OVA with or without CT.

DISCUSSION

The important and interesting findings in this study are that antigen-specific IgA antibody-producing B cells in the NALT and NP were induced and that antigen-specific IgA antibody activity was detected in nasal washings, even though Tg-mice

Table 1. Cytokines production of nasal washing. INF-γ, IL-4, IL-5, IL-6 and IL-13 production (pg/ml) from the nasal washing as determined by the ELISA method. Values represent mean in duplicate ELISA assays of pooled cell suspensions from ten (0 and 1 week) or five mice (2, 3 and 4 week).

	BALB/c mice										TCR-Tg mice									
	OVA alone						OVA+CT						OVA alone						OVA+CT					
	IFN-γ	IL-4	IL-5	IL-6	IL-13		IFN-γ	IL-4	IL-5	IL-6	IL-13		IFN-γ	IL-4	IL-5	IL-6	IL-13		IFN-γ	IL-4	IL-5	IL-6	IL-13	
0 weeks	0	0	0	0	0		0	0	0	0	0		0	0	0	0	0		0	0	0	0	0	
1 weeks	0	0	0	0	0		0	0	0	0	0		14	0	0	0	0		23	19	0	0	0	
2 weeks	0	0	0	0	0		0	0	0	0	0		44	13	0	0	5		59	24	0	13	15	
3 weeks	0	0	0	0	0		0	0	0	0	0		3	35	0	0	0		7	38	0	0	0	
4 weeks	0	0	0	0	0		0	0	0	0	0		5	24	0	0	0		5	13	0	0	0	

Table 2. IFN-γ production of NALT, NP, CLN and SP lymphocytes. INF-Á production (pg/ml) from the NALT, NP, CLN, and SP lymphocyte cells as determined by the ELISA method. Values represent mean in duplicate ELISA assays of pooled cell suspensions from ten (0 and 1 week) or five mice (2, 3 and 4 week).

	BALB/c mice								TCR-Tg mice							
	OVA alone				OVA+CT				OVA alone				OVA+CT			
	NALT	NP	CLN	SP	NALT	NP	CLN	SP	NALT	NP	CLN	SP	NALT	NP	CLN	SP
0 weeks	0	0	0	0	0	0	0	0	7	5	61	250	7	5	61	250
1 weeks	0	0	0	0	97	84	19	25	33	33	92	97	72	83	127	236
2 weeks	0	0	0	0	391	1933	1457	105	498	1870	3052	2596	447	2356	11885	2342
3 weeks	0	0	0	0	39	397	12	0	17	8	18	1391	46	2061	775	2106
4 weeks	0	0	0	0	0	0	0	0	26	17	2	296	10	120	64	357

Table 3. IL-4 production of NALT, NP, CLN and SP lymphocytes. IL-4 production (pg/ml) from the NALT, NP, CLN, and SP lymphocyte cells as determined by the ELISA method. Values represent mean in duplicate ELISA assays of pooled cell suspensions from ten (0 and 1 week) or five mice (2, 3 and 4 week).

	BALB/c mice								TCR-Tg mice							
	OVA alone				OVA+CT				OVA alone				OVA+CT			
	NALT	NP	CLN	SP	NALT	NP	CLN	SP	NALT	NP	CLN	SP	NALT	NP	CLN	SP
0 weeks	0	0	0	0	0	0	0	0	0	5	7	36	0	5	7	36
1 weeks	0	0	0	0	0	0	0	0	0	17	31	86	0	38	31	57
2 weeks	0	0	0	0	0	12	0	0	0	213	321	119	0	126	69	74
3 weeks	0	0	0	0	0	0	1	0	0	20	10	36	0	42	22	105
4 weeks	0	0	0	0	0	0	0	0	0	5	1	27	3	27	21	42

Table 4. IL-5 production of NALT, NP, CLN and SP lymphocytes. IL-5 production (pg/ml) from the NALT, NP, CLN, and SP lymphocyte cells as determined by the ELISA method. Values represent mean in duplicate ELISA assays of pooled cell suspensions from ten (0 and 1 week) or five mice (2, 3 and 4 week).

	BALB/c mice								TCR-Tg mice							
	OVA alone				OVA+CT				OVA alone				OVA+CT			
	NALT	NP	CLN	SP	NALT	NP	CLN	SP	NALT	NP	CLN	SP	NALT	NP	CLN	SP
0 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2 weeks	0	0	0	0	0	0	0	0	0	88	122	68	0	32	2	3
3 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 5. IL-6 production of NALT, NP, CLN and SP lymphocytes. IL-6 production (pg/ml) from the NALT, NP, CLN, and SP lymphocyte cells as determined by the ELISA method. Values represent mean in duplicate ELISA assays of pooled cell suspensions from ten (0 and 1 week) or five mice (2, 3 and 4 week).

	BALB/c mice								TCR-Tg mice							
	OVA alone				OVA+CT				OVA alone				OVA+CT			
	NALT	NP	CLN	SP	NALT	NP	CLN	SP	NALT	NP	CLN	SP	NALT	NP	CLN	SP
0 weeks	0	0	0	0	0	0	0	0	0	17	1	13	0	17	1	13
1 weeks	0	0	0	0	0	0	0	0	0	10	0	37	0	143	0	41
2 weeks	0	0	0	0	0	48	12	38	0	271	365	270	0	338	716	357
3 weeks	0	0	0	0	0	0	0	0	0	10	18	222	0	276	35	351
4 weeks	0	0	0	0	0	0	0	0	0	11	0	23	0	56	1	26

Table 6. IL-13 production of NALT, NP, CLN and SP lymphocytes. IL-13 production (pg/ml) from the NALT, NP, CLN, and SP lymphocyte cells as determined by the ELISA method. Values represent mean in duplicate ELISA assays of pooled cell suspensions from ten (0 and 1 week) or five mice (2, 3 and 4 week).

	BALB/c mice								TCR-Tg mice							
	OVA alone				OVA+CT				OVA alone				OVA+CT			
	NALT	NP	CLN	SP	NALT	NP	CLN	SP	NALT	NP	CLN	SP	NALT	NP	CLN	SP
0 weeks	0	0	0	0	0	0	0	0	0	11	4	28	0	11	4	28
1 weeks	0	0	0	0	0	0	0	0	0	11	13	43	0	52	26	81
2 weeks	0	0	0	0	0	4	26	121	0	239	402	340	0	241	506	206
3 weeks	0	0	0	0	0	0	3	0	0	10	5	102	0	34	71	140
4 weeks	0	0	0	0	0	0	0	0	0	11	5	58	0	11	11	71

were intranasally immunized with OVA without CT (mucosal adjuvant). These findings strongly suggest that $CD4^+$ $CD8^-$ helper T cells play an important role in assisting antigen-specific IgA and IgG production of B cells in nasopharygeal mucosa, by way of Th1 and Th2 cytokine production [14]. From this point of view, our results indicate that the frequency of antigen-specific helper T cells is the key factor to mount the significant local IgA and IgG response in the nasopharyx.

The Tg-mice (OVA-23-3) employed in this study are transgenic mice in which OVA-specific TCR genes had been introduced. Naive SP cells obtained from these mice actually show a strong proliferative response in vitro against the first stimulation with OVA, and a delayed type hypersensitivity reaction was seen following a single injection of OVA into the footpads of these mice [13]. These unique features of such mice are very advantageous to see the augmented important role of abundant OVA-specific T cells to respond to OVA in the nasopharyngeal mucosa. OVA is generally considered and used as a food allergen, but has never been used as an inhalant allergen nor as components of invading microorganisms. However, in this experiment, we employed OVA as a protein antigen in conjunction with TCR-transgenic mice (OVA23-3), in which we can evaluate the exact role of helper T cells to respond to OVA upon the same antigen stimulation in nasal mucosa, without any modification with mucosal adjuvants.

The present experiments were carried out to determine the role of antigen-specific helper T cells in mucosal IgA and IgG responses of the nasopharynx in the absence of mucosal adjuvants and to see the kinetics of mucosal IgA and IgG responses upon repeated intranasal antigen challenges by employing OVA as soluble antigen and Tg-mice as responders. The volume of antigen solution introduced into the nostril was limited to 2µl in this experiment, so as to prevent antigen entry to the lower respiratory tract as much as possible. It is well known that an intranasal immunization with antigen without cholera toxin, as well as systemic immunization, can induce a small degree of antigen-specific IgG antibodies in serum but no IgA antibodies in nasal washings. However, this IgG response is quite low, in comparison with intraperitoneal or intaradermal systemic immunization of antigens.
As a result, in Tg-mice, less OVA-specific IgA antibody-producing B cells were seen in the NALT rather than in the NP, and the production of Th2 type cytokines (IL-4, IL-5, IL-6 and IL-13) in the NALT cells was not detected. NALT in rodents works as an inductive site and the nasal passage (nasal submucosal tissue) as an effector site. However, in another investigator's report, there are 25% of Th2 type cells and 75% of Th0 type cells in among the T cell cells of murine NALT [10]. This indicates that undifferentiated, naïve T cell cells (Th0 cells) are mainly present in NALT. In comparison, T cells in the murine nasal mucosa (nasal passage) are almost all activated and differentiated to Th2 cells. These lymphocytes migrate into the nasal passage through the common mucosal immunity system (CMIS), by way of the so called lymphocyte homing to the peripheral antigen-loaded site after they had been activated with antigen at an inductive site. As we already described in the introduction, the murine NALT can be considered as the equivalent to the human Waldeyer's ring tonsils, such as the adenoid tissue. In human studies, both Th2 as well as Th1 cells are abundant in human nasal mucosa.

Among the NP cells, a large number of OVA-specific IgA antibody-producing B cells and production of IL-4, IL-5, IL-6 and IL-13 were detected. These results are in agreement with the general concept that the NALT and NP respectively act as an inductive site and an effector site in the mucosal IgA response in the upper respiratory tract. However, the existence of OVA-specific IgA antibody-producing B cells in the NALT might suggest that the NALT could be considered an effector site for the clonal expansion and differentiation of antigen-specific IgA antibody-producing B cells.
Moreover, much greater OVA-specific IgA antibody activity was found in nasal washings obtained from Tg-mice than from wild-type mice. In the Tg-mice, the levels of the Th2 type cytokines such as IL-4, IL-5, IL-6 and IL-13 peaked in the second week after nasal immunization, the number of OVA-specific IgA antibody-producing B cells in the NP peaked in the second to fourth week after the intranasal immunization, and the OVA-specific IgA antibody activity in the nasal washings peaked in the fourth to fifth week after the intranasal immunization. These findings could be explained to be the result of the induction of IgA-producing B cells by the Th2 type cytokines released from antigen-specific helper T cells mobilized in the nasal mucosa. Moreover, the total number of cells in the NALT at the second week after the intranasal immunization increased 2 to 3-times compared to that before the intranasal immunization (data not shown).
This increase might be considered due to a mobilization of lymphocytes and macrophages via lymphatic or blood vessels, and due to a clonal expansion of antigen-specific T and B cells. The number of cells in the CLN had also increased greatly in the first and second weeks after intranasal immunization compared to that before the intranasal immunization. Particularly, CLN cells of Tg-mice intranasally immunized with OVA and CT, increased in number, changing from $1x10^5$ before the intranasal immunization to $7.5x10^6$ in the second week and $8.0x10^7$ in the third week after the intranasal immunization (data not shown). These findings suggest that the primed Th0 cells in the NALT might have differentiated to Th1 and Th2 types of $CD4^+$ helper T cells in an antigen-specific fashion and that they had migrated together with IgA precursor B cells primed in the NALT to distant effecter sites such as the nasal passage via the common mucosal immunity system (CMIS) and consequently, they mounted a significant IgA response.

It was found that Th2 cytokines (IL-4, IL-6 and IL-13) were pro-

duced in the NP cells of Tg-mice when they were co-cultured in vitro with OVA without the intranasal immunization (0 week). It was also found that cytokine levels of IL-4, IL-5, IL-6 and IL-13 in the culture supernatants of NP cells sampled from intranasally immunized Tg-mice were similar to or even higher than those of SP. Furthermore, changes in time in the levels of these cytokines of NP cells were similar to those of SP. Notably, IL-6 was produced earlier and in larger amounts in NP rather than those in CLN and the levels were maintained longer in NP than in CLN.

In the Tg-mice, the numbers of OVA-specific IgA-producing B cells were higher at any interval after the intranasal immunization in the NALT than those in the CLN and SP. Moreover, the numbers of OVA-specific IgA-producing B cells were higher in the NP than those in the NALT. These results proved that the intranasal immunization with 2μl of OVA solution into the nostril actually induces a stronger local IgA immune response in the nasal mucosa compared with the lower systemic response found in SP. In addition, these data may explain the possibility that IgA-producing precursor B-1 cells in the NP, as an effecter site, were stimulated with the antigen in the nasal mucosa, and locally differentiated to the matured IgA-producing plasma cells, being independent from homing through the common mucosal immunity system (CMIS-independent IgA response) [2,3].

The above results indicate that IgA production in the nasal mucosa can be divided into two types: the CMIS-dependent type (i.e., acquired immunity) and the CMIS-independent type (i.e., innate immunity). These two types of IgA response seem to be suitable for the nasal mucosa to function as a mucosal barrier by mounting a quick and effective IgA response against a large number of invading pathogens to the nasopharyngeal mucosal linings.

To provoke an antigen-specific IgA response in the nasal mucosa of wild type mice, such as the BALB/c strain, a potent mucosal adjuvant like CT must be intranasally introduced together with the antigen, as many investigators have already reported [11,15]. CT itself is antigenic, and it has been reported that administration of CT alone into the nasal cavity induced a CT-specific IgA antibody response [15]. In the present study, we have attested successfully to provoke a local IgA response in the nasal mucosa of Tg-mice without administration of CT. No remarkable differences were found in cytokine profiles of NALT, NP, and CLN cells, numbers of antibody-producing B cells in NALT and NP, and antibody activities in nasal washings between two different groups of mice: Tg-mice intranasally immunized with OVA and CT, and those with OVA without CT. However, OVA-specific IgA and IgG activities in nasal washings, and IgA and IgG antibody-producing B cells in NP and NALT cells of Tg-mice were slightly augmented when those mice were intranasally immunized with CT, in comparison with those in mice immunized without CT. CT has been demonstrated to be a promising tool as a mucosal adjuvant for activating mucosal immunity. It has been suggested that cholera toxin enhances the retention of introduced antigens to the mucosal sites, the function of antigen-presenting cells, cytokine production from Th2 cells, and induction of apoptosis of CD8 positive T cells [16,17]. However, its toxicity remains to be fully investigated before the clinical application of a nasal vaccine in humans. The development of non-toxic mutant CT [18] or alternatively, the development of a new drug accelerating the clonal expansion of antigen-specific helper T cells in mucosal sites might enable an induction of efficient mucosal IgA and IgG responses in the nasopharynx. We also measured an antigen-specific IgE response following intranasal immunization in this study, but an OVA-specific IgE titer in nasal washings was undetectable. Generally speaking, to provoke an high enough IgE response in sera or nasal washings of mice an immunization with alum is needed. In other reports where intranasal immunization of antigens with cholera toxin or some other mucosal adjuvant was employed, antigen-specific IgE responses were reporteded [19]. However, a newly developed mutant or recombinant CT has been reported to induce no or a very low IgE production after intranasal immunization with the antigen [20,21]. The main issue (IgA reseponse) in this study is different than the type-I allergic reaction.

As it is described in this article, we have conducted animal experiments in Tg-mice, to elucidate the exact mechanism of the mucosal antibody response in the nasopharynx. Taking all data into consideration, it can be concluded that helper T cells recruited into the nasal mucosa and/or locally activated in an antigen-specific fashion are essential for mounting antigen-specific IgA and IgG responses. In view of the anatomical difference in the existence of NALT and tonsils between rodents and humans, the results obtained from our animal experiments cannot be directly extrapolated to the human situations. However, further precise studies of the mucosal immune response in the nasopharynx are still required, by employing animal models such as these Tg-mice expressing OVA-specific TCR receptor α and β, chain genes used in this study and various cytokine-knockout mice. These studies surely provide valuable information from the T cell aspect and various cytokines for the development of mucosal vaccines against infectious diseases and for the development of mucosal immunotherapies for allergic disorders [22-24].

REFERENCES.

1. McGhee JR, Mestecky J, Elson CO, Kiyono H (1989) Regulation of IgA synthesis and immune response by T cells and interleukins. J Clin Immunol 9: 175-199.
2. McGhee JR, Mestecky J, Dertzbaugh MT, Eldridge JH, Hirasawa M, Kiyono H (1992) The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 10: 75-88.
3. Kiyono H, Kweon MN, Hiroi T, Takahashi I (2001) The mucosal immune system: from specialized immune defense to inflammation and allergy. Acta Odontol Scand 59: 145-153.
4. Kiyono H (2001) Mucosal immune system: close encounter in the uncharted world of immunology. Ophthalmologica 215: 22-32.
5. Korsrud FR, Brandtzaeg P (1980) Immune systems of human

nasopharyngeal and palatine tonsils: histomorphometry of lymphoid components and quantification of immunoglobulin-producing cells in health and disease. Clin Exp Immunol 39: 361-370
6. Ichimiya I, Kawauchi H, Fujiyoshi T, Tanaka T, Mogi G (1991) Distribution of immunocompetent cells in normal nasal mucosa: comparisons among germ-free, specific pathogen-free, and conventional mice. Ann Otol Rhinol Laryngol 100: 638-642.
7. Spit BJ, Hendriksen EG, Bruijntjes JP, Kuper CF (1989) Nasal lymphoid tissue in the rat. Cell Tissue Res 255: 193-198.
8. Kuper CF, Koornstra PJ, Hameleers DM, Biewenga J, Spit BJ, Duijvestijn AM , van Breda Vriesman PJ, Sminia T (1992) The role of nasopharyngeal lymphoid tissue. Immunol Today 13: 219-224.
9. Mair TS, Batten EH, Stokes CR, Bourne FJ (1987) The histological features of the immune system of the equine respiratory tract. J Comp Pathol 97: 575-586.
10. Hiroi T, Iwatani K, Iijima H, Kodama S, Yanagita M, Kiyono H (1998) Nasal immune system: distinctive Th0 and Th1/Th2 type environments in murine nasal-associated lymphoid tissues and nasal passage, respectively. Eur J Immunol 28: 3346-3353.
11. Kawauchi H, Sano K, Ishimitsu R (1997) Mucosal Immuniity of nasopharynx: an experimental study in mice. Monduzzi Ed: tore S. P. A., Bologna, Italy: 1717-1720.
12. Kawauchi H, Sano K, Shiba H, Sano C, Yamada T, Suzuki T (2000) T-cell mucosal immunity in the nasopharynx. New Frontiers in Immunobiology, Kugler Publications, The Hague, The Netherlands: pp35-41.
13. Sato T, Sasahara T, Nakamura Y, Osaki T, Hasegawa T, Tadakuma T, Arata Y, Kumagai Y, Katsuki M, Habu S (1994) Naive T cells can mediate delayed-type hypersensitivity response in T cell receptor transgenic mice. Eur J Immunol 24: 1512-1516.
14. Kurono Y, Yamamoto M, Fujihashi K, Kodama S, Suzuki M, Mogi G, McGhee JR, Kiyono H (1999) Nasal immunization induces Haemophilus influenzae-specific Th1 and Th2 responses with mucosal IgA and systemic IgG antibodies for protective immunity. J Infect Dis 180: 122-132.
15. Fujihashi K, Yamamoto M, McGhee JR, Kiyono H (1994) Function of alpha beta TCR+ and gamma delta TCR+ IELs for the gastrointestinal immune response. Int Rev Immunol 11: 1-14.
16. Li TK, Fox BS (1996) Cholera toxin B subunit binding to an antigen-presenting cell directly co-stimulates cytokine production from a T cell clone. Int Immunol 8: 1849-1856.
17. Yankelevich B, Soldatenkov VA, Hodgson J, Polotsky AJ, Creswell K, Mazumder A (1996) Differential induction of programmed cell death in CD8+ and CD4+ T cells by the B subunit of cholera toxin. Cell Immunol 168: 229-234.
18. Yamamoto M, Briles DE, Yamamoto S, Ohmura M, Kiyono H, McGhee JR (1998) A nontoxic adjuvant for mucosal immunity to pneumococcal surface protein A. J Immunol 161: 4115-4121.
19. Tamura S, Shoji Y, Hasiguchi K, Aizawa C, Kurata T (1994) Effects of cholera toxin adjuvant on IgE antibody response to orally or nasally administered ovalbumin. Vaccine 12: 1238-1240.
20. Kweon MN, Yamamoto M, Watanabe F, Tamura S, Van Ginkel FW, Miyauchi A, Takagi H, Takeda Y, Hamabata T, Fujihashi K, McGhee JR, Kiyono H (2002) A nontoxic chimeric enterotoxin adjuvant induces protective immunity in both mucosal and systemic compartments with reduced IgE antibodies. J Infect Dis 186: 1261-1269.
21. Isaka M, Yasuda Y, Kozuka S, Taniguchi T, Miura Y, Matano K, Goto N, Tochikubo K (1999) Intranasal or subcutaneous coadministration of recombinant cholera toxin B subunit stimulates only a slight or no level of the specific IgE response in mice to tetanus toxoid. Vaccine 17: 944-948.
22. Hofstra CL, Van Ark I, Hofman G, Nijkamp FP, Jardieu PM, Van Oosterhout AJ (1998) Differential effects of endogenous and exogenous interferon-gamma on immunoglobulin E, cellular infiltration, and airway responsiveness in a murine model of allergic asthma. Am J Respir Cell Mol Biol 19: 826-835.
23. Huang TJ, Macary PA, Kemeny DM, Chung KF (1999) Effect of CD8+ T-cell depletion on bronchial hyper-responsiveness and inflammation in sensitized and allergen-exposed Brown-Norway rats. Immunol 96: 416-423.
24. Ishimitsu R, Nishimura H, Yajima T, Watase T, Kawauchi H, Yoshikai Y. (2001) Overexpression of IL-15 in vivo enhances Tc1 response, which inhibits allergic inflammation in a murine model of asthma. J Immunol 166: 1991-2001.

Hideyuki Kawauchi, MD, DMSc
Department of Otolaryngology
Shimane University
89-1, Enya-cho
Izumo City
Japan

Tel: +81-853-20-2273
Fax: +81-853-20-2271
E-mail: kawauchi@shimane-med.ac.jp