ORIGINAL

Defect of oral tolerance in NC/Nga mice

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Abstract : The NC/Nga mouse is a model animal for human atopic dermatitis. In this study, we investigated oral tolerance induction in NC/Nga mice. In BALB/c mice, oral administration of ovalbumin (OVA) resulted in suppression of both OVA-specific T and B cell responses induced by OVA immunization. In NC/Nga mice, OVA-induced antigen (Ag) -specific T and B cell responses were significantly less than those in BALB/c mice. Furthermore, oral administration of OVA did not suppress OVA-specific immunoresponses in NC/Nga mice. We further examined antibody (Ab) response against food Ag by feeding mice an experimental diet that contained OVA or casein as a protein source. The level of serum OVA or casein-specific IgG was significantly higher in NC/Nga mice than in BALB/c mice. These results indicate that NC/Nga mice have a defect in the induction of oral tolerance. NC/Nga mice can therefore be used as a model for investigating the mechanism of oral tolerance. J. Med. Invest. 53 : 29-33, February, 2006

Keywords **:** *NC/Nga mice, oral tolerance, antigen, antibody, ovalbumin*

INTRODUCTION

Immunologic tolerance has been defined as a mechanism by which the immune system prevents pathologic autoreactivity against self-antigens (Ags) and thus prevents autoimmune diseases. Induction and maintenance of Ag-specific unresponsiveness and/or suppression in peripheral T cells is an effective way to preventing or curing immune disorders, such as allergies, autoimmne diseases and inflammation induced during transplantation (1-3). Oral tolerance has classically been defined as the specific suppression of cellular and/or humoral immune responses to an Ag by prior administration of the Ag by the oral route. It presumably evolved to prevent hypersensitivity reactions to food proteins and bacterial Ags present in the mucosal flora (4). Breakdown of oral tolerance might disrupt immunosuppressive mechanisms, resulting in development of immune disorders. Indeed, children who are atopic are less likely to develop oral tolerance to ingested foods and as a result are more likely to have manifestations of food allergy, including wheezing, skin rashes and gastrointestinal symptoms (5).

Atopic dermatitis is one of the most common skin diseases in children with a family history of atopy (6) and is frequently associated with elevated serum levels of IgE antibodies (Abs) against many kinds of inhaled allergens (7). NC/Nga mice have been shown to develop spontaneous severe dermatitis when kept in conventional conditions (8). The NC/Nga mouse has been used as a model animal for studies on allergies. The mechanisms of the onset of dermatitis have not been clarified, but it has been suggested that immunological factors contribute to its progress (9,10). In this study, we focused on oral tolerance and investigated its induction in NC/Nga mice.

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MATERIALS AND METHODS

Mice, Ag administration and immunization

Eight-week-old NC/Nga and BALB/c mice were purchased from SLC (Hamamatsu, Japan) and maintained in the Animal Experimental Center of Tokushima University under specific-pathogen free conditions. Mice were administered 5mg of ovalubumin (OVA) by intragastric gavage for 5 connective days. Control mice were administered PBS instead of OVA. After oral administration of Ag, mice were immunized with 10 μg OVA conjugated with 1 mg of aluminun hydroxide (HCI Biosector, Denmark) intraperitoneally 2 times at a 2-week interval.

This study conformed to the guidelines for the care and use of laboratory animals of Institution of Health Bioscience, The University of Tokushima Graduate School, Tokushima, Japan.

OVA and casein diets

A diet containing OVA (Sigma, St. Louis, MO) and bovine casein (Sigma) at 20 % as the only protein source was prepared. The composition of the diet is shown in Table 1. The diets were begun when mice were 8 weeks old and continued for 60 days.

Determination of anti-OVA and anti-casein Ab levels in serum

Serum OVA and casein-specific Ab levels were measured by ELISA using microtiter plates coated with 25 μg/ml OVA or casein. Serially diluted serum was added to the wells, incubated at room temperature for 2 hr, and then washed with PBS containing 0.05% Tween 20. Alkaline phosphatase (AP) - conjugated anti-mouse IgG, IgG 2 a, or IgG 1 (Southern Biotechnology Associates Inc., AL.) was diluted 1,000 - fold, added to the wells, and then incubated for 2 hr at 37℃. For the determination of IgE levels, 1,000 - fold diluted biotin-conjugated anti-mouse IgE (BD Pharmingen, San Diego, CA) was added to the wells and incubated at room temperature for 1 hr, and then 3,000 - fold diluted avidin - conjugated AP was added to the wells and incubated for 1hr. After washing, enzymatic activity was visualized using a substrate, p-nitrophosphate. OD was measured using a test wavelength of 415 nm.

Proliferation assay

Spleen cells (5×10^5) were cultured with OVA (100 μg/ml) or plate - bound anti - CD 3 mAb (2 μg/ml) in 200 μl of complete RPMI medium (supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2 - mercaptoethanol). Proliferation was assayed by [^{3}H] methyl - thymidine incorporation in the last 12 hr of a 72 hr culture period.

Stastistical analysis

Data are expressed as means±SD. The statistical significance of data was determined by Student's t-test. A p value of less than 0.05 was taken as significant.

RESULTS

In BALB/c mice treated with PBS orally, immunization with OVA induced an Ag-specific immune response as determined by *in vitro* lymphocyte proliferation response and *in vivo* Ab response. However, oral administration of OVA significantly suppressed these responses. Oral OVA treatment decreased *in vitro* proliferation response from 40, 360±6,980 to 10,058± 1,560 cpm and significantly decreased serum OVA-specific IgG, IgG 1 and IgE levels. In NC/Nga mice treated with PBS, both OVA-specific *in vitro* proliferation and *in vivo* Ab responses were significantly less than those in BALB/c mice, except for IgG 2 a production. When NC/Nga mice were administered OVA orally, suppressions of OVA - specific proliferation and Ab response were not observed (Figs. 1 and 2) . These results suggest that NC/Nga mice have a defect in induction of oral tolerance.

Next, to investigate tolerance induction under physiological conditions, we examined production of Ab against food Ag in mice fed OVA or casein diet. Mice were given OVA or casein diet containing OVA or casein as a protein source for 60 days. After receiving the experimental diet, serum was collected, and OVA and casein - specific Abs were determined. Levels of anti - OVA and anti-casein IgG were significantly increased in NC/Nga mice compared to those in BALB/c mice (Fig. 3) . Although we also determined Ag - specific IgE level, increment was not observed during the experimental period (data not shown) . This indicates that NC/Nga mice potentially produce IgG against diet -derived Ags.

DISCUSSION

Induction and maintenance of Ag - specific unresponsiveness and/or suppression in peripheral T cells is an effective way for preventing or curing immune disorders, such as allergies, autoimmne diseases and inflammation induced during transplantation (1-3) . In regarding to allergy response, both oral and nasal ad-

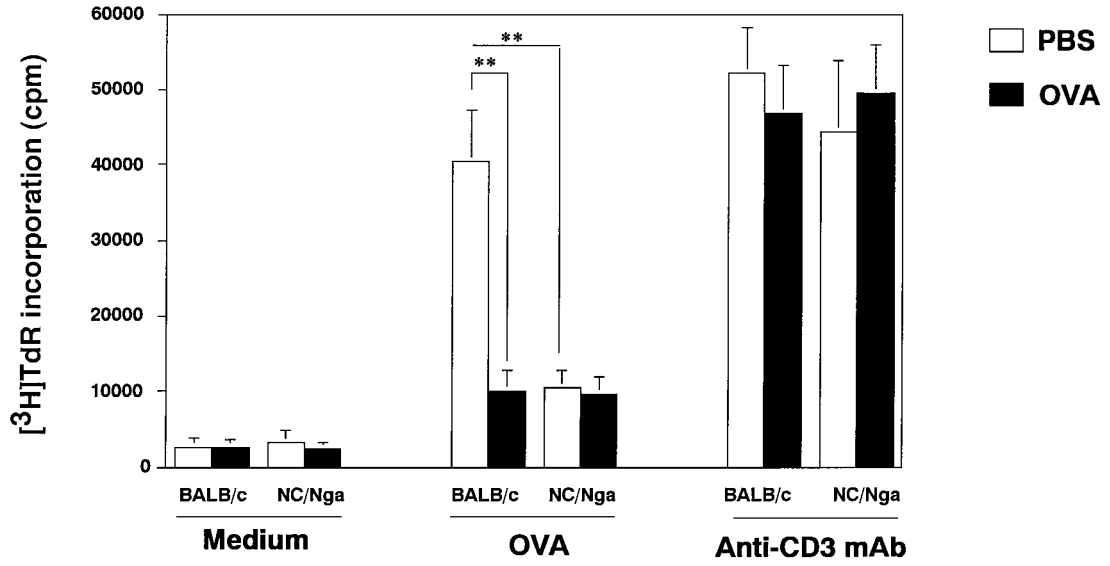

Fig. 1. Proliferation responses to OVA in BALB/c and NC/Nga mice orally administered OVA or PBS followed by immunization with OVA. Two weeks after the second immunization, spleen cells were stimulated with OVA or anti-CD 3 mAb for 72 hr. Proliferation response was determined by counting incorporated [3H] TdR activity. The results are expressed as means±SD.

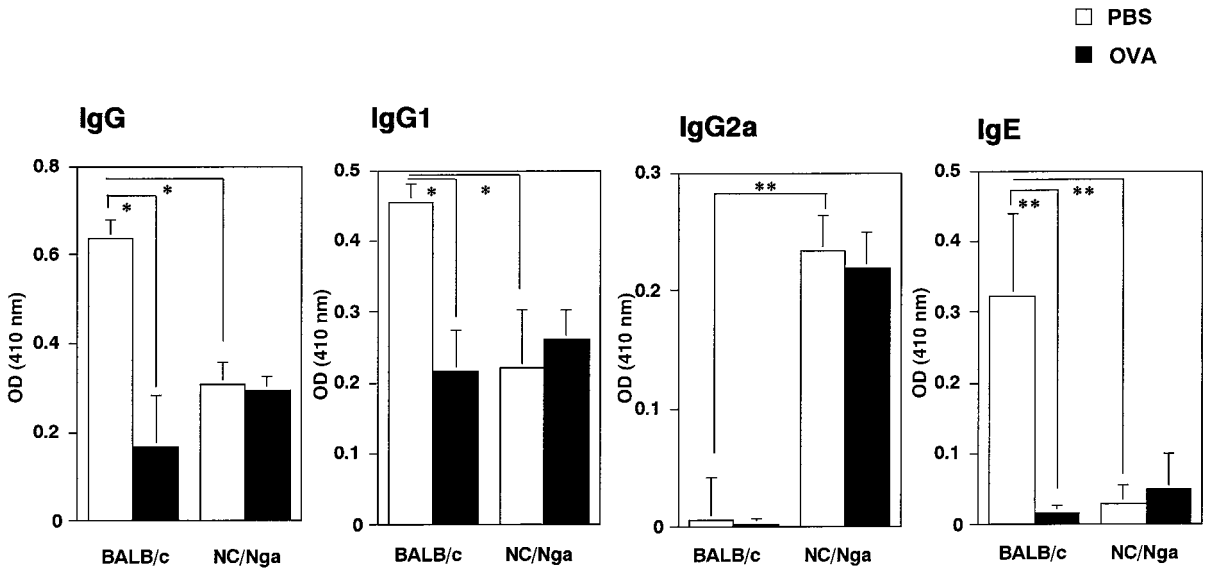

Fig. 2. Analysis of OVA-specific Ig subclasses in serum of BALB/c and NC/Nga mice orally administered OVA or PBS followed by immunization with OVA. Serum was obtained two weeks after the second immunization. The levels of OVA-specific IgG, IgG1, IgG2a and IgE were determined by ELISA. The results are expressed as means±SD.

ministration of allergens have been shown to be effective for suppressing IgE responses and intestinal mast cell responses (11). NC/Nga mice develop skin disorder with increasing serum IgE levels in conventional conditions. We examined oral tolerance induction in NC/Nga mice because maintenance of oral tolerance is crucial for prevention of allergic response. We found in this study that NC/Nga mice exhibit a defect in tolerance against orally administered Ag.

Hyporesponsive to exogenous Ag in NC/Nga mice was unexpected because NC/Nga mice have been reported to produce much higher levels of IgE than those produced in BALB/c mice in conventional conditions (8). We focused on MHC class II molecules and examined their expressions. Expression of I - A^d on $CD11c^+$ cells in NC/Nga mice were two to three - times lower than those in BALB/c mice (data not shown). MHC class II has been shown to be involved in Ag -specific CD 4^+ T cell induction and subsequent Ab responses. Indeed, MHC class II-deficient mice exhibit impaired Ab responses against trinitrophenol (TNP) (12). T and B cells from NC/Nga mice do not have potent defects in their functions. An *in vitro* proliferation assay revealed that spleen cells from NC/Nga mice

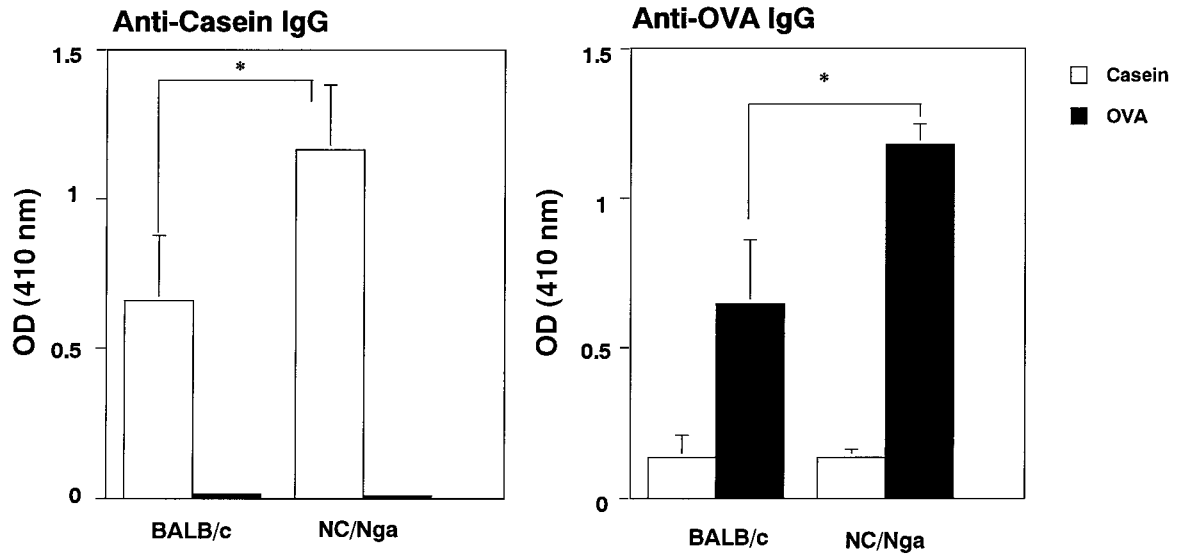

Fig. 3. Analysis of casein and OVA-specific IgG in serum of BALB/c and NC/Nga mice fed casein or OVA diet. BALB/c and NC/Nga mice were fed casein (white bars) or OVA (black bars) diet containing casein or OVA as the only protein source, respectively. After 60 days, serum was collected and casein or OVA-specific IgG level in 10-fold diluted serum was determined by ELISA.

Table 1. Composition of experimental diet.

	Groups	
	OVA	Casein
OVA	250[1]	-
Casein	-	250
Cornstarch	413	413
Sucrose	207	207
Soy bean oil	50	50
Vitamin mixture[2]	50	50
Mineral Mixture[3]	10	10
Cellulose	20	20

[1]g/kg diet.

[2]Vitamin mixure was composed of (mg or IU/kg diet) : retinal acetate, 5,000IU;ergocalciferol, 1,000IU;tocopheral acetate, 50 mg ; manadion, 52 mg ; thiamine chloride, 12 mg ; riboflavin, 40 mg ; pyridoxine-HCl, 8 mg ; vitamin B 12, 0.005 mg ; ascorbic acid, 300 mg ; D-biotin, 0.2 mg ; folic acid, 2 mg ; calcium pantothenate, 50 mg ; p-amino benzoic acid, 50 mg ; niacin, 60 mg ; inositol, 60 mg ; choline chloride, 2,000 mg.

[3]Mineral mixture was composed of (mgkg diet) : $CaHPO_4/2\ H_2O$, 7,280; KH_2PO_4, 12,860; NaH_2PO_4,4,680; NaCl, 2,330; Ca-lactate, 17,750 ; Fe-citrate, 1,590 ; $MgSO_4$, 3,590 ; $ZnCO_3$, 55 ; $MnSO_4/4\text{-}5\ H_2O$, 60 ; $CuSO_4/5\ H_2O$, 15 ; KI, 5.

normally respond to anti-CD3 stimulation (Fig. 2) . Furthermore, when NC/Nga mice were immunized with a thymus-independent Ag, TNP-Ficoll, production levels of TNP - specific IgM and IgG were same as those in BALB/c mice (data not shown) . Thus, insufficient responsiveness to exogenous Ag in NC/Nga mice might contribute to the MHC class II - dependent Ag-specific T cell induction. However, it can not exclude a possibility that defect of Ag-presenting function is responsible for low T cell responsiveness *in vitro* in NC/Nga mice.

Oral tolerance refers to systemic Ag hyporesponsiveness that occurs after oral Ag administration and can occur by multiple mechanisms. Some of the mechanisms have been elucidated. Low doses of oral antigen preferentially induce active suppression (13,14) , whereas high doses induce clonal anergy and deletion (15, 16) . Oral antigen preferentially generates a Th2 (IL-4/IL-10) - or a Th3 (TGF - β) - type response (4,11) . Th3 - type cells are a unique T cell subset that primarily secrete TGF - β, provide help for IgA and have suppressive actions on Th 1 and other immune cells. In addition, CD 4^+ $CD25^+$ regulatory T cells, which are anergic and have suppressive properties, have been shown to be induced after oral Ag administration and to be involved in oral tolerance (17,18) . In this study, we did not examine Th2, Th3 and $CD4^+$ $CD25^+$ regulatory T cell functions. Further examination of their functions is needed to elucidate the mechanism of impaired oral tolerance in NC/Nga mice.

In contrast to OVA - specific IgG, IgG 1 and IgE production, the level of specific IgG2a was significantly increased in NC/Nga mice (Fig. 2) . It has been demonstrated that IFN-γ is crucial for production of IgG2a subclass. To address this point, we determined IFN-γ production *in vitro* and found that the level of IFN-γ production was lower than that in BALB/c mice as reported previously (19) . We further examined FcγRIIB molecules that serve as a negative feedback regulator for B cell Ag receptor-elicited activation

of B cells (20) . We found deletion polymorphism in the *Fcgr 2b* promotor region in NC/Nga mice as reported in systemic autoimmune disease - prone NZB, BXSB, MRL and NOD mice (21) (T. S. and S. Y., unpublished observation) . We are currently investigating the deletion polymorphism in the *Fcgr 2 b* promotor region to Ab responses in NC/Nga mice.

The results of this study indicate NC/Nga mice will serve as a useful model for investigating oral tolerance in order to better understand the mechanisms.

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