Arch Pharm Res Vol 24, No 6, 557-563, 2001

Archives of Pharmacal Research
http://apr.psk.or.kr

Induction of Oral Tolerance to Japanese Cedar Pollen

Joung-Hoon Kim[1], Yeun-Ja Mun[1], Seong-Hun Ahn[1], Joung-Suk Park[2], and Won-Hong Woo[1]

[1]Department of Herbal Resources, Professional Graduate School of Oriental Medicine, Wonkwang University and [2]Department of Pharmacy, Graduate School of Wonkwang University, Sinyong-dong, Iksan, Chunbuk, 570-749, Korea

(Received July 25, 2001)

Oral tolerance is thought to play a role in preventing allergic responses and immune-mediated diseases. An improved mouse model of the oral tolerance to Japanese cedar pollen (JCP) as antigen was developed in order to detect induction of the tolerance, and the immunological characteristics of this model were also elucidated. Oral tolerance was induced by C3H/HeN mice given an oral administration of 10 mg JCP 7 days before immunization with an i.p. injection of 0.1 mg JCP in complete Freunds adjuvant (CFA). The effects of oral JCP on systemic immunity were assessed by enzyme-linked immunosorbent assay (ELISA) of immunoglobulin (Ig) levels in serum collected on day 7 or 14 after immunization. Oral tolerance to JCP was adequately induced on day 7 after immunization and was more effective in C3H/HeN mice than in BALB/c mice. The tolerance was primarily concerned with the decreased serum levels of antigen-specific IgG. In these mice, oral administration of JCP also suppressed various immune responses to the antigen including delayed-type hypersensitivity (DTH), total IgE level and anti-JCP IgG1 level. The suppression of these immune responses by the oral antigen was associated with a significant reduction in interleukin-4 (IL-4) production. These findings therefore indicate that this C3H/HeN mice model has potential use in detecting the induction of oral tolerance by JCP, and suggest that this tolerance model may be effective in the treatment and prevention of allergic responses caused by the antigen.

Key words: Oral tolerance, Japanese cedar pollen, Immunoglobulin subset level, Interleukin-4

INTRODUCTION

We have previously reported that experimental models for oral tolerance were developed by intraperitoneal applications of antigen in orally sensitized mice (Kim and Ahn, 1995; Kim and Ohsawa, 1995). In these models it was found that antigen plays a central role in the process leading to immunological tolerance. Namely, oral administration of a soluble protein antigen induces a state of unresponsiveness in cellular and humoral immune responses to the antigen, a process termed oral tolerance (Mowat, 1987; Weiner *et al.*, 1994). Induction of this tolerance is proposed to be an immunotherapy of allergic (Mowat, 1987) and autoimmune diseases (Higgins and Weiner, 1988; Nussenblatt *et al.*, 1990; Cooke and Wraith, 1993).

Correspondence to: Joung-Hoon Kim, Department of Herbal Resources, Professional Graduate School of Oriental Medicine, Wonkwang University
E-mail: jhunkim@wonkwnag.ac.kr

Although the physiological significance of oral tolerance still remains unclear, it is thought to play a role in preventing allergic responses to soluble protein antigens and immune-mediated diseases. For instance, it has been suggested that potential consequences of a breakdown in oral tolerance may be food-sensitive enteropathies such as celiac disease and cow's milk protein intolerance observed in infants with immature intestinal function (Kiyono *et al.*, 1988). It has also been shown that defective oral tolerance induced by immunosuppressive agents promoted nephritogenesis in experimental immunoglobulin A (IgA)-mediated nephropathy of mice (Gesualdo *et al.*, 1990). A convenient model is therefore needed to identify the tolerance in order to determine its pathophysiological role.

On the other hand, it is well recognized that allergic disorders in humans are a common symptom in IgE-mediated allergic diseases, and major sources eliciting these diseases are pollens from grasses, house dust mites, molds and Japanese cedar (*Cryptomeria japonica*) pollen (JCP) (Hoffmann-Sommergruber *et al.*, 1996). As recently

as 25 years ago, allergic disease induced by JCP was rare, but the incidence of this disease is now on the increase, caused by abundant and widespread air pollution in Japan. However, there are few reports related to the animal model for allergic disease caused by JCP (Yasuda *et al.*, 1999). It is well known that an immediate hypersensitivity reaction in allergic conjunctivitis induced by JCP in humans depends upon IgE antibody production (Ito *et al.*, 1988). These findings suggest that the detection of oral tolerance induction by JCP may be effective in treating or preventing allergic responses produced by the antigen.

The present study was attempted, therefore, to develop a convenient mouse model of the oral tolerance to JCP in order to detect induction of the tolerance within a short period and to investigate the immunological characteristics of this model.

MATERIALS AND METHODS

Animals

Male C3H/HeN and BALB/c mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). CE-2 (Clea Japan Inc., Tokyo, Japan), rodent laboratory chow, and tap water were provided *ad libitum*. Mice were used for experiments at 6 weeks of age after 7 days acclimatization.

Antigen

JCP (Torii Pharmaceutical, Tokyo, Japan) was dissolved in sterile distilled water (10 mg/ml) for oral administration or in complete Freund's adjuvant (CFA; Sigma Chemical Co. St. Louis, MO, USA) (1 mg/ml) for immunization.

Induction of oral tolerance to JCP

To induce oral tolerance, mice were given an oral administration of 10 mg JCP in 1 ml sterile distilled water. Control animals were given a corresponding volume of sterile distilled water. Seven days later they were immunized with an *i.p.* injection of 0.1 mg JCP in CFA. On either day 7 or 14 after immunization, blood samples for antibody determinations were collected by heart puncture. After centrifuging the samples, sera were collected and stored at -70°C until assay of antibody levels.

Measurement of delayed-type hypersensitivity

On day 7 after immunization, 10 μg of JCP dissolved in 20 μl of phosphate-buffered saline (PBS) was injected *s.c.* into the right mouse footpad. As a vehicle control, 20 μl of PBS was injected into the left footpad. The thickness of the right and left footpads were measured using dial gauge calipers calibrated in 0.01 mm graduations (Mitutoyo Mfg. Co., Ltd., Japan), both immediately before and 24 h after the challenge injection. The increase in left footpad thickness was subtracted from the increase in right footpad thickness to give the increase due solely to the specific response to the antigen. In unsensitized mice, responses to JCP and PBS were essentially equivalent.

Measurement of total serum IgE antibody

Total IgE levels in serum were determined by an enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann, 1972). Briefly, each well of a 96-well microplate was coated with affinity-purified goat antibody to IgE (Yamasa Shoyu Co., Ltd., Tokyo, Japan) by adding 50 μl of the antibody solution (10 μg/ml PBS) and incubating overnight at 4°C. Each well was then washed with PBS containing 0.05% Tween (PBS-Tween) and blocked by the addition of 150 μl of 1% bovine serum albumin (BSA)/PBS, followed by incubation for 2 h at room temperature. After extensive washing of the wells with PBS-Tween, 50 μl aliquots of appropriately diluted serum sample or standard IgE monoclonal antibody (Yamasa Shoyu) were added to each well. The microplate was then incubated at room temperature for 2 h. It was again washed as described above. Biotinylated monoclonal anti-mouse IgE (Serotec, Oxford, U.K.) in BSA/PBS was added to each well (100 ng/50 μl/well). The plate was incubated at room temperature for 2 h. After incubation and extensive washing, the wells were probed with 50 ml aliquots of avidin-horseradish peroxidase (HRP) (E-Y Laboratories Inc., San Mateo, CA, diluted 1:300 with PBS), followed by further incubation for 1 h in darkness at room temperature. After extensive washing, peroxidase activities were assayed as follows: 100 μl of substrate solution (10 mg of *o*-phenylenediamine and 10 μl of 30% H_2O_2 in 25 ml of 0.1 M citrate-phosphate buffer, pH 5) was added to each well of the plate. The plates were incubated for 15 min at room temperature, and the enzyme reaction was terminated by the addition of 100 μl of 2 N H_2SO_4 per well. The optical density (OD) at 490 nm of each well was then measured with a microplate spectrophotometer (Sunnyvale, CA, USA). The OD values of blanks containing no serum were subtracted from those of the standards and unknowns. The concentrations of the various samples were obtained by interpolation on standard curves. The final concentration of each sample was calculated by averaging the results of duplicate cultures.

Measurement of antigen-specific IgG, IgG1 and IgG2a antibodies

Anti-JCP IgG, IgG1 and IgG2a antibodies were measured with an ELISA (Engvall and Perlmann, 1972). Anti-JCP IgG2a and IgG1 levels were used as indicators for functions of two Th subpopulations, Th1 and Th2 cells, respectively, which differentially regulate B cells (Miller and Hanson, 1979; Challacombe and Tomasi, 1980; Michalek *et al.*, 1992). For ELISA of anti-JCP antibodies, wells of a 96-

well microplate were coated with 100 μl/well of JCP (100 mg/ml) dissolved in PBS and incubated at 4°C for 2 h. After washing the microplate three times with PBS-Tween, the wells were blocked with BSA/PBS for 2 h at room temperature. To obtain an OD reading on a linear curve from the serial dilutions, the serum samples were diluted with BSA/PBS at a ratio of 1/40 for IgG and IgG2a measurements and at 1/10 for IgG1 measurement. One hundred micro-liter of each sample was added to the plate well and incubated at room temperature for 1 h before washing again. Aliquots of 100 μl of HRP-conjugated goat anti-mouse IgG (Caltag, Burligame, CA, USA), IgG1, or IgG2a (PharMingen, San Diego, CA, USA) diluted with BSA/PBS were added to each plate. The plates were further incubated for 1 h at room temperature. After washing, peroxidase activities were assayed according to the procedures of ELISA for anti-JCP Ig levels. Enzyme reaction was terminated by the addition of 50 μl of 1 N H_2SO_4 to each well. The concentrations of each anti-JCP Ig subclass were obtained by interpolation on the standard curves.

Cytokine measurement

To induce the serum cytokines, mice receiving oral administration of JCP were challenged 2 h before assay with 200 μg of lipopolysaccharide (Sigma) dissolved in 200 μl of saline intravenously. Blood samples for cytokines were collected by cardiac puncture of the mice. The separated serum was stored frozen at -70°C until assayed. Cytokine production was quantified using sandwich ELISA techniques. In brief, wells of a 96-well microtiter plate were coated overnight at 4°C with 100 μl/well of 0.1 M phosphate buffer containing antibodies (4 μg/ml) against IFN-γ and IL-4 (PharMingen). The plates were blocked by incubation with 150 μl/well of BSA/PBS at 37°C for 1 h. Washed three times, and samples or standards (recombinant mouse IFN-γ and IL-4; PharMingen) were added to each well to a volume of 100 μl and incubated at 37°C for 1 h. The plates were washed three times and 100 μl/well of biotinylated anti-mouse IFN-γ and IL-4 (2 μg/ml) antibodies (PharMingen) diluted in BSA/PBS was added. After incubation at 37°C for 1 h, the plates were washed three times, and 100 μl/well of streptavidin-alkaline phosphatase (PharMingen) was added at 2 μg/ml. The plates were washed again before 100 μl of *p*-nitrophenylphosphase was added to each well. The plates were then read at 405 nm using a microplate spectrophotometer. Cytokine levels were determined with reference to a standard curve constructed using serial dilutions of the standard cytokines, and results were expressed in pg/ml.

Statistical analysis

The values were expressed as mean ± standard error (S. E.). All data were examined for their statistical significance of difference with Student's *t* test.

RESULTS

Standardization for the induction of oral tolerance to JCP

Firstly an investigation was carried out to establish convenient conditions for the induction of oral tolerance to JCP in mice. C3H/HeN mice given an oral administration of 10 mg JCP were immunized 7 d later with an i.p. injection of 0.1 mg JCP in CFA. When compared with controls, the JCP-fed mice showed marked suppression of anti-JCP IgG and total IgE levels in serum collected on day 7 after the immunization. The suppression was still retained on day 14 but to a lesser degree than that on day 7 (Table I). Thus, the induction of oral tolerance to JCP can be detected adequately on day 7 after the immunization and most significantly on anti-JCP IgG levels.

Using the anti-JCP IgG levels as an indicator for oral tolerance to JCP, it was further examined whether the induction of oral tolerance in C3H/HeN mice differed from that in the BALB/c mice which are generally recognized as being susceptible to such induction. As shown in Table II, BALB/c mice fed 10 mg JCP 7 d before immunization also displayed significant suppression of their serum anti-JCP IgG levels compared with controls. However, the suppression (down to 73.9% of control value) was much less than that in C3H/HeN mice (down to 42.5% of control value). These findings indicate that oral tolerance to JCP was expressed on day 7 after immunization and was more effective in C3H/HeN mice than in BALB/c mice, an increase in effectiveness associated mainly with decreased anti-JCP IgG.

Table I. Time course for induction of oral tolerance to JCP in C3H/HeN mice

Group	Day 7		Day 14	
	Anti-JCP IgG (OD)	Total IgE (μg/ml)	Anti-JCP IgG (OD)	Total IgE (μg/ml)
Control	1.20 ± 0.19	3.94 ± 0.38	0.94 ± 0.17	4.13 ± 0.57
JCP	0.5 1 ± 0.06**	2.85 ± 0.15*	0.52 ± 0.08*	3.78 ± 0.40

C3/HeN mice (JCP group) were fed 10 mg JCP by gavage. Control mice were fed distilled water alone. All mice were immunized with 0.1 mg JCP in CFA i.p. 7 d after JCP feeding. On day 7 or 14 after immunization, total IgE and anti-JCP IgG levels in serum were measured using ELISA. Antibody levels of day 7 and 14 sera were assayed at the same dilution. Results represent the mean ± S.E. of 5 or 6 mice. Asterisks denote a significant difference compared to control. *P<0.05, **P <0.01.

Table II. Mouse strain variation in serum levels of antigen-specific IgG in oral tolerance to JCP

Strain	Group	Anti-JCP IgG (OD)	% of control
C3H/HeN	Control	1.20 ± 0.19	42.5
	JCP	0.51 ± 0.06**	
BALB/c	Control	0.69 ± 0.05	73.9
	JCP	0.51 ± 0.05*	

Mice of JCP group were fed 10 mg JCP by gavage. Control mice were fed distilled water alone. All mice were immunized with 0.1 mg JCP in CFA i.p. 7 d after JCP feeding. Seven days after immunization, serum anti-JCP IgG levels were measured using ELISA. Results represent the mean ± S.E. of 5 or 6 mice. Asterisks denote a significant difference compared to control mice. *P< 0.05, **P<0.01.

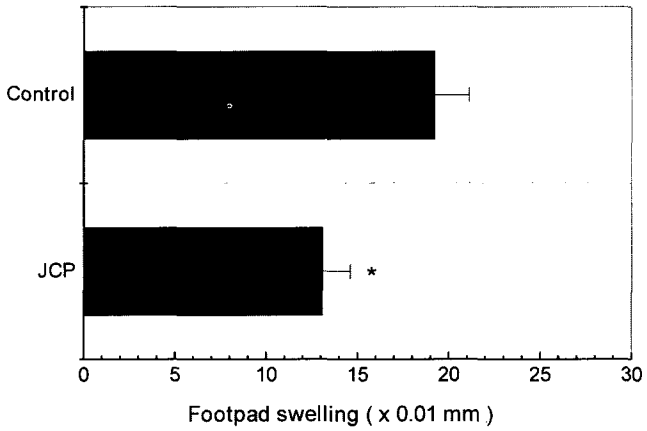

Fig. 1. The suppression of DTH responses to JCP in C3H/HeN mice by antigen ingestion. Mice were fed 10 mg JCP 7 d before immunizing with JCP in CFA, and footpad-tested 7 d after immunization. Two groups of mice were given an oral administration of distilled water (control) or 10 mg JCP (JCP). Each column represents the mean ± S.E. of results obtained from 5 or 6 mice. Statistical significance from control was set at *P<0.05.

DTH responses to JCP by feeding the antigen

To learn whether JCP suppresses cell-mediated immune responses to the antigen in C3H/HeN mice, DTH to JCP was induced in the footpad of antigen fed. As shown in Fig. 1, mice fed 10 mg JCP were observed to undergo significant suppression of DTH response to JCP (0.19 ± 0.19 mm, P<0.05), as compared with controls (0.13 ± 0.02 mm). This finding indicates that JCP has significant tolerance of footpad DTH response, associated with its T cell-mediated response.

Serum levels of Anti-JCP IgG subclasses following JCP

The suppression of humoral immune responses to JCP was investigated by measuring levels of antigen-specific IgG subclasses in C3H/HeN mice receiving oral JCP prior to antigen immunization. Since mice fed 10 mg JCP exhibited marked suppression of ant-JCP IgG antibody response

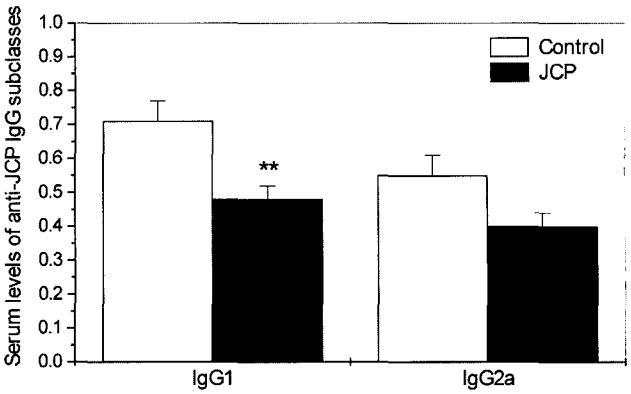

Fig. 2. The serum levels of anti-JCP IgG subclasses in C3H/HeN mice by JCP. All mice groups were immunized with JCP in CFA as described in Table II. Seven days before immunization, two groups of mice were given an oral administration of distilled water (control) or 10 mg JCP (JCP). Serum antibodies were measured using ELISA. Each column represents the mean ± S.E. of duplicate samples from 5 or 6 mice. Statistical significance from control was set at **P<0.01.

compared with control mice (1.20 ± 0.19, vs. 0.51 ± 0.06, respectively, P<0.01) (Table II), the levels of anti-JCP IgG2a and IgG1 antibodies were measured to investigate the effects of orally administered JCP on Th1 (Burnstein and Abbas, 1993) and Th2 (Isakson *et al.*, 1982) cell responses, respectively. The results are shown in Fig. 2. Greatly reduced production of anti-JCP IgG1 antibody was observed in JCP-fed mice compared with control mice (0.71 ± 0.06, vs. 0.48 ± 0.04, respectively, P<0.01). Although a similar pattern was also observed in anti-JCP IgG2a antibody, the reduction was not significantly different from controls. The above results thus indicate that JCP exhibits a marked suppression of humoral immune response such as anti-JCP IgG1 levels.

The secretion of INF-γ and IL-4 by feeding JCP

The levels of INF-γ and IL-4, also known as Th1 and Th2 cytokines (Diamantstein *et al.*, 1988; Mu and Sewell, 1994), respectively, were measured as well to examine whether the suppression of immune responses to JCP in mice was associated with Th1 and Th2 types of $CD4^+$ T cell responses. JCP feeding significantly suppressed IL-4 (down to 61.7% of control value) as shown in Fig. 3. A moderate decrease of INF-γ production was also observed in JCP-fed animals compared with controls. Therefore, oral JCP is also likely to strongly suppress Th cell function, in particular Th2 cell function.

DISCUSSION

The present study was undertaken to develop a convenient mouse model of the oral tolerance to JCP in

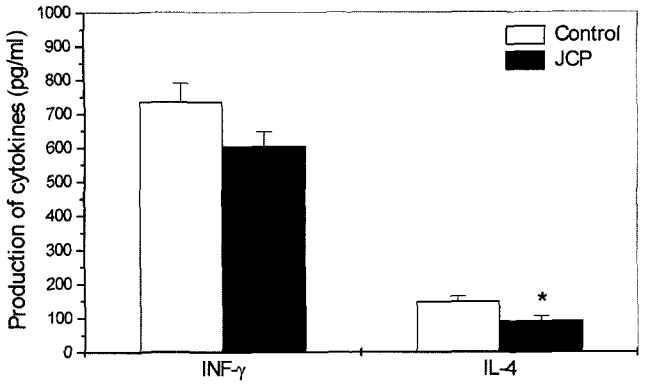

Fig. 3. The suppression of cytokine secretion in C3H/HeN mice by JCP ingestion. All mice groups were immunized with JCP in CFA as described in Table II. Seven days before immunization, two groups of mice were given an oral administration of distilled water (control) or 10 mg JCP (JCP). IFN-γ and IL-4 contents were measured by sandwich ELISA as described in *Materials and Methods*. Each column represents the mean ± S.E. of duplicate samples from 5 or 6 mice. Statistical significance from control was set at $*P<0.05$.

order to detect induction of the tolerance within a short period and to investigate the immunological characteristics of this model.

Several experimental schedules have been described for the induction of oral tolerance to soluble protein antigens in mice. For instance, it has been shown that feeding high doses (more than 5 mg) of antigen appeared to induce anergy (Friedman and Weiner, 1994). Hanson and Miller (1982) also found that oral administration of antigen was effective in inducing oral tolerance to the antigen in mice. In addition, similar results were seen in our own previous studies (Kim and Ahn, 1995; Kim and Ohsawa, 1995). Our preliminary study (data not shown) of JCP showed that as little as 1 mg of JCP fed to C3H/HeN mice on alternate days for 2 weeks was sufficient to induce tolerance. Moreover, oral tolerance in mice fed 10 mg JCP was expressed adequately by day 7 after immunization and was more effective in C3H/HeN mice than in BALB/c mice, an increase in effectiveness associated mainly with decreased anti-JCP IgG (Table II). Thus, we used a single orally administered dose of 10 mg JCP to C3H/HeN animals in order to induce oral tolerance.

It has been suggested that oral tolerance induces B cell anergy, and the production of tolerogens by a mechanism such as suppressor T (Ts) cell activation and/or anergy of Th cells (Mowat, 1987). The evidence implying Ts cells activation has been obtained in studies on mice (Mattingly and Waksman, 1978; Ngan and Kind, 1978; Mowat, 1994). However, a number of recent studies have shown that oral tolerance focused mainly on defective Th cell function rather than on active Ts cells (Melamed and Friedman, 1993; Fishman-Lobell *et al.*, 1994; Garside *et al.*, 1995; Melamed *et al.*, 1996). It has been demonstrated that murine Th1 cells mediate DTH *via* IL-2 production and regulate IgG2a production *via* IFN-γ production, whereas Th2 cells regulate IgG1 and IgE production *via* IL-4, IL-5, IL-10 and IL-13 production (Snapper and Paul, 1987; Mosmann and Coffman, 1989; Paul, 1989; Pond *et al.*, 1989; Takano *et al.*, 1997). In addition, our previous study established that anti-ovalbumin IgG level was more sensitive in its tolerance than total serum IgG and IgE levels (Kim and Ohsawa, 1995). Similarly, the present study also confirmed that the induction of oral tolerance was more effective in anti-JCP IgG antibody level than in total serum IgE level (Table I). It was further revealed that the feeding of JCP significantly suppressed DTH response to the antigen, antigen-specific IgG subclasses and cytokines production in serum (Figs. 1-3). To our knowledge, this is the first report of the induction of oral tolerance by JCP. Furthermore, since recent study of this tolerance has focused mainly on defective Th cell functions, the results of the present study on Th cell function are presented as follows.

Th1 cells, a subset of $CD4^+$ T cells, have been shown to play a role in the induction of DTH and IgG2a antibody production *via* IL-2 and IFN-γ production, respectively (Th1 responses) (Boom *et al.*, 1988; Fong and Mosmann, 1989). In agreement with these findings, feeding JCP decreased DTH response to the antigen as compared with those in controls (Fig. 1) but was not effective in inhibiting the secretion of IFN-g and IgG2a antibody in JCP-fed C3H/HeN mice (Figs. 2 and 3). Thus, all these findings suggest that feeding JCP may not induce Th1 oral tolerance.

Th2 cells regulate IgG1 and IgE production *via* IL-4, IL-5, IL-10 and IL-13 production (Snapper and Paul, 1987; Mosmann and Coffman, 1989; Paul, 1989; Pond *et al.*, 1989). Similar to the Th1 function, the feeding of JCP to mice significantly suppressed Th2 function as demonstrated by the significant decreases of IL-4 production (Fig. 3) as well as of anti-JCP IgG1 and total IgE levels in serum (Table I, Fig. 2). These findings were similar to that previously reported of a single high dose of antigens reducing the production of antigen-specific IgG1 antibody (Garside *et al.*, 1995; Kim and Ohsawa, 1995). Therefore, oral JCP in the present study was confirmed to result in the marked suppression of Th2 cell responses. To clarify the link between oral tolerance and allergic response to JCP, further studies are required to analyze the precise mechanism by which JCP promotes IgE specific suppressor T (Ts) cell activation, and to investigate the differences in mechanism according to animal species.

In conclusion, feeding JCP seems to be effective in inducing oral tolerance to the antigen including DTH response, antigen-specific IgG1 antibody production and IL-4 secretion in C3H/HeN mice. These findings therefore indicate that this model has potential use in detecting the induction of tolerance to a specific antigen.

ACKNOWLEDGEMENTS

This work was supported by the Brain Korea 21 Project, and partially by Wonkwang University in 2001.

REFERENCES

Boom, W. H., Liano, D., and Abbas, A. K., Heterogeneity of helper/inducer T lymphocytes. II. Effects of interleukin-4- and interleukin-2-producing T cell clones on resting B lymphocytes. *J. Exp. Med.*, 167, 1350-1363 (1988).

Burnstein, H. J. M. and Abbas, A. K., *In vivo* role of interleukin-4 in T cell tolerance induced by aqueous protein antigen. *J. Exp. Med.*, 177, 457-463 (1993).

Challacombe, S. J. and Tomasi, T. B., Systemic tolerance and secretory immunity after oral immunization. *J. Exp. Med.*, 152, 1459-1472 (1980).

Cooke, A. and Wraith, D. C., Immunotherapy of autoimmune disease. *Curr. Opin. Immunol.*, 5, 925-933 (1993).

Diamantstein, T., Eckert, R., Volk, H. D., and Kupier-Weglinski, J. W., Reversal by interferon-gamma of inhibition of delayed-type hypersensitivity induction by anti-CD4 or anti-interleukin-2 receptor (CD25) monoclonal antibodies. Evidence for the physiological role of the $CD4^+$ $Th1^+$ subset in mice. *Eur. J. Immunol.*, 18, 2101-2103 (1988).

Engvall, E. and Perlmann, P., Enzyme-linked immunosorbent assay. III. Quantitation of specific antibodies of enzyme labeled anti-immunoglobulin in antigen-coated tubes. *J. Immunol.*, 109, 129-135 (1972).

Fishman-Lobell, J., Friedman, A., and Weiner, H. L., Different kinetic patterns of cytokine gene expression *in vivo* in orally tolerant mice. *Eur. J. Immunol.*, 24, 2720-2724 (1994).

Fong, T. A. and Mosmann, T. R., The role of interferon-gamma in delayed-type hypersensitivity mediated by Th1 clones. *J. Immunol.*, 143, 2887-2893 (1989).

Friedman, A. and Weiner, H. L., Induction of energy or active suppression following oral tolerance is determined by antigen dosage. *Proc. Natl. Acad. Sci. USA*, 91, 6688-6692 (1994).

Garside, P., Steel, M., Worthey, E. A., Satoskar, A., Alexander, J., Bluethmann, H., Liew, F. Y., and Mowat, A. M., T helper 2 cells are subject to high dose oral tolerance and are not essential for its induction. *J. Immunol.*, 154, 5649-5655 (1995).

Gesualdo, L., Lamm, M. E., and Emancipator, S. N., Defective oral tolerance promotes nephritogenesis in experimental IgA nephropathy induced by oral immunization. *J. Immunol.*, 145, 3684-3691 (1990).

Hanson, D. G. and Miller, S. D., Inhibition of specific immune responses by feeding protein antigens. III. Induction of the tolerant state in the absence specific suppressor T cells. *J. Immunol.*, 128, 2378-2393 (1982).

Higgins, P. and Weiner, H. L., Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein and its fragments. *J. Immunol.*, 140, 440-445 (1988).

Hoffmann-Sommergruber, K., Ferreira, E. D., Ebner, C., Barisani, T., Korninger, L., Kraft, D., Scheiner, O., and Baumgartner, I., Detection of allergen-specific IgE in tears of grass pollen-allergic patients with allergic rhinoconjunctivitis. *Clin. Exp. Allergy*, 26, 79-87 (1996).

Isakson, P. C., Pure, E., Vitetta, S., and Krammer, P. H., T cell-derived B cell differentiation factor(s): effect on the isotype switch of murine B cells. *J. Exp. Med.*, 155, 734-748 (1982).

Ito, K., Ishii, A., Yamashita, N., Miyamoto, T., and Watanabe, N., Comparison of guinea pig IgE antibodies estimated by ELISA with those estimated by passive cutaneous anaphylaxis. *Int. Arch. Allergy Appl. Immunol.*, 87, 424-429 (1988).

Kim, J. H. and Ahn, Y. K., Effects of diphenyl dimethyl dicarboxylate on oral tolerance to ovalbumin in mice. *J. Toxicol. Sci.*, 20, 375-382 (1995).

Kim, J. H. and Ohsawa, M., Oral tolerance to ovalbumin in mice as a model for detecting modulators of the immunologic tolerance to a specific antigen. *Biol. Pharm. Bull.*, 18, 854-858 (1995).

Kiyono, H., Green, D. R., and McGhee, J. R., Contrasuppression in the mucosal immune system. *Immunol. Res.*, 7, 67-81 (1988).

Mattingly, J. A. and Waksman, B. H., Immunological suppression after oral administration of antigen. I. Specific suppressor cells formed in rat Peyers patches after oral administration of sheep erythrocytes and their systemic migration. *J. Immunol.*, 121, 1878-1883 (1978).

Melamed, D. and Friedman, A., Direct evidence for anergy in T-lymphocytes tolerized by oral administration of ovalbumin. *Eur. J. Immunol.*, 23, 935-942 (1993).

Melamed, D., Fishman-Lobell, J., Uni, Z., Weiner, H. L., and Friedman, A., Peripheral tolerance of Th2 lymphocytes induced by continuous feeding of ovalbumin. *Int. Immunol.*, 8, 717-724 (1996).

Michalek, S. M., Kiyono, H., Wannemuehler, M. J., Mosteller, L. M., and McGhee, J. R., Lipopolysaccharide (LPS) regulation of the immune response: LPS influence on oral tolerance induction. *J. Immunol.*, 128, 1992-1998 (1982).

Miller, S. D. and Hanson, D. G., Inhibition of specific immune responses by feeding proteins. IV. Evidence for tolerance and specific active suppression of cell-mediated immune responses to ovalbumin. *J. Immunol.*, 123, 2344-2350 (1979).

Mosmann, T. R. and Coffman, R. L., Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.*, 7, 145-173 (1989).

Mowat, A. M., Oral tolerance and regulation of immunity

to dietary antigens. In Ogra, P. L., Mestecky, J., Lann, M. E., Strober, W., McGhee, J. R. and Bienesstock, J. (Eds.). *Handbook of Mucosal Immunology.* pp. 185-210, Academic Press, San Diego. CA, (1994).

Mowat, A. M., The regulation of immune responses to dietary protein antigens. *Immunol. Today,* 8, 93-95 (1987).

Mu, H. H. and Sewell, W. A., Regulation of DTH and IgE responses by IL-4 and IFN-γ in immunized mice given pertussis toxin. *Immunology,* 83, 639-645 (1994).

Ngan, J. and Kind, L. S., Suppressor T cells for IgE and IgG in Peyers patches of mice made tolerant by the oral administration of ovalbumin. *Immunology,* 120, 861-865 (1978).

Nussenblatt, R. B., Caspi, R. R., Mahdi, R., Chan, C., Roberge, F., Lider, O., and Weiner, H. L., Inhibition of S-antigen induced experimental autoimmune uveoretinitis by oral induction of tolerance with S-antigen. *J. Immunol.,* 144, 1689-1695 (1990).

Paul, W. E., Pleiotropy and redundancy: T cell derived lymphokine in the immune response. *Cell,* 57, 521-540 (1989).

Pond, L., Wasson, D. L., and Hayes, C., Evidence for differential induction of helper T cell subset during *Trichinella spiralis* infection. *J. Immunol.,* 143, 4232-4237 (1989).

Snapper, C. M. and Paul, W. E., Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate immunoglobulin isotype production. *Science,* 236, 944-947 (1987).

Takano, H., Yoshikawa, T., Ichinose, T., Miyabara, Y., Imaoka, K., and Sagai, M., Diesel exhaust particles enhance antigen-induced airway inflammation and local cytokine expression in mice. *Am. J. Respir. Crit. Care Med.,* 156, 36-42 (1997).

Weiner, H. L., Friedman, A., Miller, A., Khoury, S. J., Al-Sabbagh, A., Santos, L., Sayegh, M., Nussenblatt, R. B., Trentham, D. E., and Hafler, D. A., Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens. *Annu. Rev. Immunol.,* 12, 809-837 (1994).

Yasuda, M., Kato, M., Nakata, K., and Kohno, S., An experimental allergic conjunctivitis induced by topical and repetitive applications of Japanese cedar pollens in guinea pigs. *Inflamm. Res.,* 48, 325-336 (1999).